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13. ABSTRACT (Maximum 200 Words)
The goal of the present proposal is to provide post-doctoral training opportunities in breast cancer research that focus on the role of microenvironment in mammary gland biology. Trainees will benefit from working in a dynamic interactive program under the guidance of the LBNL mentors to investigate the intersection of hormone action, growth factor activity and extracellular matrix remodeling during mammary gland development and carcinogenesis. In addition, trainees will be exposed to a variety of other topics related to breast cancer, as well as research ranging form molecular medicine to genomics, by their participation in working groups, lectures and scientific meetings with other Berkeley Lab and Bay Area researchers.

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Introduction

The goal of the present proposal is to provide post-doctoral training opportunities in breast cancer research that focus on the role of microenvironment in mammary gland biology. Trainees will benefit from working in a dynamic interactive program under the guidance of the LBNL mentors to investigate the intersection of hormone action, growth factor activity and extracellular matrix remodeling during mammary gland development and carcinogenesis. In addition, trainees will be exposed to a variety of other topics related to breast cancer, as well as research ranging from molecular medicine to genomics, by their participation in working groups, lectures and scientific meetings with other Berkeley Lab and Bay Area researchers.

Recruitment

As reported previously, applicants for the postdoctoral fellowships were recruited by posting advertisements at major cancer meetings, on mammary biology lists and through the Lawrence Berkeley National Laboratory Human Resources webpage. In additional, personal contacts and unsolicited applicants to individual mentors were considered for these positions.

- American Association of Cancer Research posting
- LBNL web posting: Initial #13392, Ongoing #13447; Web address: http:cjo.lbl.gov/
- Posted to Mammary Biology List (maintained by P. Neville at the University of Colorado)

As reported previously approximately 20 applicants responded. Their letters and CVs were circulated to the mentor groups. Mentors then indicated their enthusiasm for individual applicants, who were invited to LBNL to present their current research. Mentors interviewed applicants and discussed possible research projects. Candidates were informed at the time that their research projects would be the result of collaboration between at least two mentors. Three candidates were recruited. Of the original recruits, Rana Zahedi obtained independent funding from the Department of Defense Breast Cancer Research program, Scott Jepson works in a biotechnology firm in the United Kingdom and Norisa Uehara has just recently left to take an academic position in Japan. The current trainees are Anna Erickson and Joanna E. Mroczkowska-Jasinska, who replaced Scott Jepson in 2002. We will begin to recruit for the third postdoctoral fellow this fall.

Key Research Accomplishments

Postdoctoral Fellows

- Anna Erickson, Ph.D.

Dr. Erickson joined the Barcellos-Hoff lab in June 2001 to conduct research on a joint project with Dr. Barcellos-Hoff and Bissell. She obtained her degree in Cell Biology from the University of Alabama at Birmingham in 2001 in the laboratory of Dr. John Couchman. She has studied E-cadherin mRNA, protein abundance and localization, and its association with other membrane and cytoskeletal proteins in cells surviving radiation exposure in the three-dimensional model of alveolar morphogenesis (See Appendix 3, Abstract 1). In part, this project was funded by DOD in 2001 to Dr. Barcellos-Hoff under the title "Basis of Persistent Microenvironment Perturbation in IR Human Mammary Epithelial Cells". Additional support was obtained by Dr. Barcellos-Hoff through the Department of Energy (DOE) Low Dose

Radiation Program. Dr. Erickson also supervised an undergraduate from the University of California, Berkeley over the last 18 months, who assisted her with studies on centrosomes. An abstract of these data will be presented at meetings this fall (See Appendix 3, Abstract 2), and a manuscript is being prepared.

Dr. Erickson and Dr. Barcellos-Hoff published a review for Expert Opinion in Therapeutic Targets (See attached publication) on the utility of therapeutic targeting of stroma and extracellular matrix in breast cancer. She is currently writing a draft manuscript that will report the studies on E-cadherin localization. She was involved in studies which showed that ionizing radiation induces heritable disruptions of epithelial cell interactions (See attached publication). She has presented her research findings at the Era of Hope Meeting in Orlando, Florida in September 2002, and will be presenting at the American Society of Cell Biology in December, 2003 in San Francisco, and the AACR Special Conference on TGF-β in January, 2003.

- Joanna E. Mroczkowska-Jasinska, Ph.D.

Dr. Mroczkowska-Jasinska joined the lab of Drs. Paul Yaswen and Martha Stampfer in June 2002. Joanna is using human mammary epithelial cells (HMEC) to study pathways that influence telomerase expression in human mammary epithelial cells during immortalization. In particular, she is examining the effects of perturbations in three transcription factors; p53, ZNF217 and BORIS, on telomerase expression and immortalization. It had previously been reported by the Yaswen/Stampfer group that inactivation of p53 function can induce expression of telomerase activity in early passage "conditionally" immortal HMEC with low or undetectable levels of telomerase. Employing promoter reporter assays, Joanna has been able to demonstrate higher activity of the hTERT promoter in conditionally immortal HMEC exposed to a dominant negative inhibitor of p53 function. These studies provide evidence that the suppression of telomerase by p53 is transcriptional. Further studies to localize the p53 responsive element(s) in the hTERT promoter are currently in progress. In collaboration with Dr. Jean Benhattar at the Institut Universitaire de Pathologie in Switzerland, Joanna has also been investigating the degree of correlation between hTERT promoter methylation and hTERT transcription level in HMEC at different stages of immortalization. Joanna has performed quantitative RT-PCR and has found that higher hTERT mRNA levels correlate with increased methylation of the hTERT promoter in HMEC immortalized with the ZNF217 oncogene. Another potential oncogene, BORIS, has been reported to have a role in reprogramming the methylation pattern of particular tracts of DNA, including sites in the hTERT gene. Joanna is using retroviral constructs to determine whether exogenous BORIS expression extends the proliferative potential of, or immortalizes finite life span HMEC. Early preliminary results suggest that up-regulated BORIS expression can indeed extend the life span of certain HMEC. Further phenotypic analyses of cells transduced with BORIS are under way. This work was recently submitted to be presented at a Keystone meeting this winter (See Appendix 3, Abstract 3).

- Norisa Uehara, Ph.D.

Dr. Uehara joined Dr. Shyamala's laboratory in April 2001 and was appointed on the training grant in December 2001. He left LBNL in April of 2003 to take a faculty position in Japan. The focus of his research was on the role of progesterone receptors in mammary development and

neoplasia using various genetically engineered mouse models. In these studies he characterized the epithelial cells in the mammary glands of progesterone A (PR-A) transgenics which have been shown to exhibit extensive ductal growth, loss in basement membrane integrity and cell–cell adhesion, characteristics commonly associated with transformed cells. Dr. Uehara worked with Dr. Yu-Chien Chou to show that the expression patterns of p21, ER[{alpha}] and cyclin D1 are altered in the mammary epithelial cells of PR-A transgenics and are accompanied by a higher rate of proliferation as revealed by immunostaining for BrdU and PCNA. This work resulted in a publication in Carcinogenesis in March of 2003. (See attached publication) In addition, Dr. Uehara looked at the localization of an inhibitor of differentiation/DNA binding (Id-1) which has been shown to promote proliferation and to inhibit functional differentiation of mouse mammary epithelial cells (SCp2 cells), maintained in cell culture. Dr. Uehara found that Id-1 was not localized to the luminal epithelial cells in vivo regardless of the strain or the developmental stage, instead he found that it was myoepithelial cell-specific markers, which contribute towards our current understanding of the biology of mammary myoepithelial cells. This work resulted in a publication Breast Cancer Res in Jan. of 2003. (See attached publication)

Training Activities

The trainees are exposed to a wide range of research approaches, tools, and methods that are encompassed in the mentor's laboratories. In addition to weekly **laboratory meetings** with the preceptor, a monthly **Cell and Molecular Biology department meeting** is held to bring together the investigators and the trainees to discuss research and literature relevant to the program. The department will host a Postdoctoral Research Day on December 5 that will feature poster presentations and a speaker chosen by postdoctoral fellows. **Division seminars** are held weekly (see Appendix 1 consisting of a roster of speakers for 2003-2004).

Of particular relevance is the monthly **Mammary Gland Affinity Group**, which is a long standing tradition. LBNL mammary biology and breast cancer groups meet for informal research presentations. Additional participants from UC San Francisco Medial Center and UC Berkeley campus attend regularly. Approximately 30-40 participate. The format consists of two short talks by postdoctoral fellows.

The Life Sciences Division currently hosts approximately 50 research grants in breast cancer and mammary biology, totaling over $16 million in funds. Dr. Mina Bissell, the Principal Investigator of the Training Grant, recently stepped down as the director of the Life Sciences Division to devote more time to breast cancer research. She is the recipient of an Innovator Award from the DOD Breast Cancer Research Program. We are happy to report that Dr. Joe Gray has taken this directorship. Dr. Gray maintains a joint appointment with the UCSF Cancer Center where he is the program leader of the Breast Oncology Program. This program contains s the NCI-funded Bay Area Breast Cancer Specialized Program Of Research Excellence (SPORE). The Breast Oncology Program has a weekly seminar series which we now video conference to LBNL. These seminars provide the postdoctoral fellows with a good mixture of basic research and clinical research. It also provides a good opportunity for the postdoctoral fellows to hear and understand the concerns of breast cancer advocates. (See Appendix 2)

Reportable Outcomes

The work described above as resulted in four publications and presentations in three meetings.(see attachments below)

Conclusions

The postdoctoral fellows being trained under this grant are successfully learning how to design and perform meaningful research which adds to the basic understanding of mechanisms involved in breast cancer. Their work has resulted in several peer reviewed publications and presentations and we anticipate several more in the coming year.

References

1 - Chou, Y. C., **Uehara, N.**, Lowry, J. R., and Shyamala, G. (2003). Mammary epithelial cells of PR-A transgenic mice exhibit distinct alterations in gene expression and growth potential associated with transformation. Carcinogenesis *24*, 403-409.

2 - **Erickson, A. C.**, and Barcellos-Hoff, M. H. (2003). The not-so innocent bystander: the microenvironment as a therapeutic target in cancer. Expert Opin Ther Targets *7*, 71-88.

3 - Park, C. C., Henshall-Powell, R. L., **Erickson, A. C.**, Talhouk, R., Parvin, B., Bissell, M. J., and Barcellos-Hoff, M. H. (2003). Ionizing radiation induces heritable disruption of epithelial cell interactions. Proc Natl Acad Sci U S A *100*, 10728-10733.

4 - **Uehara, N.**, Chou, Y. C., Galvez, J. J., de-Candia, P., Cardiff, R. D., Benezra, R., and Shyamala, G. (2003). Id-1 is not expressed in the luminal epithelial cells of mammary glands. Breast Cancer Res *5*, R25-29.

Please see attached abstract and publications.

APPENDIX #1

Life Science Division Seminar Series 2003/2004

Date	Speaker	Affiliation
Sep 16, 2003	*Joe Gray	Life Sciences Division Director, LBNL
Sept 23, 2003	Ronald DePinho	Dana Farber
Oct 7, 2003	Neal Copeland	National Cancer Institute
Oct 14, 2003	Bradley Rice	Xenogen Corporation
Oct 21, 2003	Thomas Lindahl	CR-UK Clare Hall Laboratories
Oct 28, 2003	Frank McCormick	UCSF
Nov 4, 2003	Bruce Hasegawa	UCSF
Nov 11, 2003	W. James Nelson	Stanford University
Nov 18, 2003	Andre Nussenzweig	National Cancer Institute
Nov 25, 2003	Wolfgang Baumeister	Max-Planck Institute of Biochemistry
Dec 2, 2003	Thomas Cooper	Baylor College of Medicine
Dec 9, 2003	Thomas Lewellen	University of Washington Medical Center
Dec 16, 2003	Gary Stormo	Washington University St. Louis
	2004	
Jan 6, 2004	Lee Edwin Goldstein	Massachusetts General Hospital
Jan 13, 2004	Stuart Kim	Stanford University
Jan 16, 2004	K. Namba	Osaka University
Jan 20, 2004	Teresa Head-Gordon	University of California, Berkeley
Jan 22, 2004	*Elaine Fuchs	HHMI - The Rockefeller University
Jan 27, 2004	Hugo Bellen	Baylor College of Medicine
Feb 3, 2004	Shiv Grewal	Cold Spring Harbor Laboratory
Feb 10, 2004	Cynthia McMurray	Mayo Medical School
Feb 17, 2004	Huntington Willard	Institute for Genome Sciences & Policy
Feb 24, 2004	TBD	
March 2, 2004	Victor Lobanenkov	LIP NIAID NIH

March 9, 2004	*Phillip Sharp	Massachusetts Institute of Technology
March 16, 2004	David Drubin	University of California Berkeley
March 23, 2004	Joel Karp	University of Pennsylvania
March 30, 2004	Caroline Tanner	The Parkinson's Institute
April 6, 2004	Nancy Bonini	HHMI – University of Pennsylvania
April 13, 2004	William Muller	Royal Victoria Hospital
April 20, 2004	TBD	
April 27, 2004	Scott Fraser	California Institute of Technology
May 3, 2004	*Joan Massague	HHMI – Memorial Sloan-Kettering
May 4, 2004	Pamela Bjorkman	California Institute of Technology
May 11, 2004	Michael Kastan	St. Jude Children's Research Hospital
May 18, 2004	Nikolaus Grigorieff	HHMI - Brandeis University
May 25, 2004	William Brinkley	Baylor College of Medicine

* Denotes Distinguished Speaker

APPENDIX #2

Breast Oncology Program seminar series 2003

Date	Speaker	Affiliation
Sept. 10, 2003	William Goodson and Dan Moore	California Pacific Medical Research Institute
Sept. 17, 2003	Mark Sternlicht	UCSF
Sept. 24, 2003	Myles Brown	Dana Farber Cancer Institute - Harvard
Oct. 1, 2003	Joe Gray	UCSF/LBNL
October 8	Bob Hiatt	UCSF
October 15	TBA	
October 22	Jeff Tice	UCSF
October 29	Mark Moasser	UCSF
Nov. 12, 2003	Priscilla Cooper	Lawrence Berkeley National Lab
Nov. 19, 2003	Paul Yaswen	Lawrence Berkeley National Lab
Nov. 26, 2003	David Stokoe	SPORE Developmental Project
Dec. 10, 2003	Thea Tlsty	UCSF
Dec. 17, 2003	Elizabeth Blackburn	UCSF

Appendix 3

ABSTRACT #1

American Society for Cell Biology, 2003

Radiation Exposure Promotes TGF-β Induced EMT in Human Mammary Epithelial Cells

Anna C. Erickson, William S. Chou, Mina J. Bissell and Mary Helen Barcellos-Hoff

Department of Cell and Molecular Biology, Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, California 94720

We have shown that transforming growth factor- β1 (TGF- β1) is rapidly activated in response to ionizing radiation (IR) in mouse mammary gland and mediates epithelial cell fate decisions after radiation. Here, we used pre-malignant S1 HMT-3522 human mammary epithelial cells (HMEC) to characterize their response to TGF-β and IR. Consistent with reports for other epithelial cell lines, S1 monolayers show a dose dependent (0.2-1.2 ng/ml) response to TGF- β characterized by decreased E-cadherin and β-catenin, induction of fibronectin, and inhibition of growth. Irradiated S1 cells exhibit increased nuclear SMAD, suggesting an increase in TGF-β signaling, and reduced levels of E-cadherin and β-catenin that was restored with TGF-β neutralizing antibody treatment. Thus, as in mouse, human cells respond to IR by activating TGF-β. To mimic the presence of irradiated stroma, irradiated cells were grown in the presence of additional TGF-β, which did not augment loss of E-cadherin and β-catenin, but did result in significantly decreased E-cadherin immunofluorescence. To determine the nature of this alteration, we used differential detergent extraction of soluble proteins followed by immunoprecipitation or immunofluorescence. E-cadherin and β-catenin cytoskeletal association was significantly reduced in irradiated, TGF-β 1 treated cells compared to either irradiated or TGF- β1 treated cells. Irradiated, TGF- β1 treated cells also exhibited increased vimentin immunofluorescence and protein abundance. These features are consistent with epithelial-mesenchymal transition (EMT), and suggest that IR exposure promotes EMT in pre-malignant HMECs in a manner distinct from, but augmented by, that induced by TGF- β1 signaling. Importantly, this effect of IR is heritable and could contribute to its action as a carcinogen in breast.

ABSTRACT #2

American Society for Cell Biology, 2003

TGF-β Protects Human Mammary Epithelial Cells from Radiation-Induced Centrosomes Amplification

Rishi Gupta, Anna C. Erickson and Mary Helen Barcellos-Hoff

Department of Cell and Molecular Biology, Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, California 94720

In recent studies, we have shown that ionizing radiation (IR), a known carcinogen of human and murine mammary gland, compromises human mammary epithelial cell (HMEC) polarity and multicellular organization in a manner characteristic of neoplastic progression through a heritable, non-mutational mechanism (Park et al., PNAS, in press). The irradiated HMEC phenotype is augmented by TGF-β,..which is rapidly activated in response to IR in mouse mammary gland and plays a critical role in epithelial cell fate decisions. Since TGF-β can either suppress or promote tumor progression via a variety of mechanisms, we asked whether TGF- β would augment the radiation-induced genomic instability in S1 cells. Centrosome defects frequently accompany tumor progression, so we examined centrosome stability in this model. Non-malignant S1 HMT-3522 HMEC were seeded as monolayers and subjected to IR 4 hours post plating. Daughters of the surviving cells were analyzed for centrosome abnormalities six days later by immunofluorescent staining for γ-tubulin. IR increased the frequency of S1 cells with 3 or more centrosomes as a function of radiation dose up to 5 Gy. TGF-β treatment (400 pg/ml) of irradiated HMEC decreased the frequency of cells with abnormal centrosomes numbers. Consistent with our studies in irradiated mice, irradiated HMEC also activate TGF-β and blocking it with TGF-β neutralizing antibodies resulted in increased centrosome amplification. Thus, TGF-β plays a dual role in response to IR by protecting against genomic instability generated by radiation-induced centrosome amplification, while promoting phenotypic neoplastic progression.

ABSTRACT #3

Proceedings of the National Academy of Sciences, 2003

Changes in hTERT gene methylation during immortalization of human mammary epithelial cells

Joanna Mroczkowska, Stéphanie Renaud, Martha Stampfer, Jean Benhattar, and Paul Yaswen

Life Sciences Div., Lawrence Berkeley National Lab, Berkeley, CA 94720 and
Institut Universitaire de Pathologie, Bugnon 25CH-1011, Lausanne, Switzerland

Repression of hTERT expression presents a stringent block to immortal and tumorigenic transformation of most human somatic cells *in vitro* and *in vivo*. To define a pathologically relevant model of human mammary epithelial cell (HMEC) immortalization, we have exposed cells cultured from normal tissue to a variety of potential immortalizing agents, e.g., chemical carcinogens, oncogenes (c-*MYC*, *ZNF217*), and inhibitors of p53 function. Each of these agents, alone and in combination, can induce immortalization and hTERT expression, albeit inefficiently and indirectly. To determine whether additional epigenetic changes might be involved, we have measured changes in the methylation status of the hTERT promoter in HMEC before, during, and after immortalization using a quantitative methylation-sensitive dot blot assay and methylation-sensitive single-strand conformation analysis. Analysis of a cell line immortalized by exposure to *ZNF217* indicates that the hTERT promoter becomes fully methylated as these p53(+) cells gradually acquire increased telomerase activity. This methylation does not occur in one step, but slowly and heterogeneously with increasing passage. The mechanism responsible for this progressive methylation and derepression of hTERT is under investigation.

Carcinogenesis vol.24 no.3 pp.403–409, 2003

Mammary epithelial cells of PR-A transgenic mice exhibit distinct alterations in gene expression and growth potential associated with transformation

Yu-Chien Chou, Norihisa Uehara, Jason R.Lowry and G.Shyamala[1]

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Expression of the 'A' and 'B' forms of progesterone receptor (PR), in an appropriate ratio is critical for normal mammary development. As such, mammary glands of PR-A transgenic mice, carrying additional 'A' form of PR as transgene, exhibit morphological and histological characteristics associated with transformation. Accordingly, in the present studies, we analyzed these mammary glands for the presence of transformed epithelial cells by examining for alterations in gene expressions and growth potential, known to be associated with different stages of transformation. These studies reveal that, in the aberrant mammary epithelial structures, there is a decrease in p21 expression, an increase in cyclin D1 expression accompanied by an increase in cell proliferation, and a decrease in estrogen receptor alpha (ERα). In mammary ducts with normal histology, there is a decrease in p21 expression without an elevation in cyclin D1 expression or cell proliferation or a decrease in ERα expression. Treatment of PR-A transgenics with anti-progestin, mifepristone, has no effect on cell proliferation, cyclin D1 or ERα expression in the aberrant epithelial structures. In contrast, mifepristone restored the loss of p21 expression in the epithelial cells of both the ducts with normal histology and aberrant structures. Parallel studies reveal no apparent differences between the mammary glands of wild-type and PR-B transgenic mice, which carry additional PR 'B' form. Accordingly, we conclude that (i) mammary glands of PR-A transgenics contain at least two distinct populations of transformed epithelial cells, (ii) the epithelial cell population in the ducts with normal histology contain presumptive immortalized cells, indicative of early stages of transformation, (iii) the aberrant epithelial structures contain later stages of transformation associated with hyperplasias/pre-neoplasias and (iv) the transformation of mammary epithelial cells in PR-A transgenics might be due to a misregulation in progesterone action resulting from overexpression of PR 'A' form.

Introduction

The female sex steroids, estradiol and progesterone, signaling through their respective receptors, ERα and PR are critical for normal mammary development, induction of mammary carcinogenesis and growth of some mammary tumors. A central role for PR in normal mammary development is established by the fact that in PR null mutant mice, which have ERα, there is a severe inhibition in lobulo-alveolar growth, normally accompanying pregnancy and occurring in response to estradiol and progesterone. At present the precise role of PR in mediating either normal mammary development or carcinogenesis is unknown (1,2).

PR exists in two molecular forms (the 'A' and 'B' forms) which actions can vary depending on cell and promoter context (3). Previous studies from our laboratory have shown that a regulated expression of the two isoforms of PR is critical for normal mammary development. As such, in transgenic mice carrying an imbalance in the native ratio of 'A' to 'B' forms by overexpressing either the 'A' or 'B' form (referred to as PR-A and PR-B transgenics, respectively) mammary development is abnormal (4,5). In particular, mammary glands of PR-A transgenics exhibit excessive ductal growth, contain aberrant epithelial structures with ducts composed of multiple layers of epithelial cells and a loss in basement membrane integrity and cell–cell adhesion (4), characteristics frequently associated with transformed cells.

Extensive studies by Medina *et al.* have shown that normal, pre-neoplastic and neoplastic mouse mammary epithelial cells exhibit distinct patterns of gene expression and growth properties (6–8). In our previous studies, the aberrant features associated with mammary glands of PR-A transgenic mice were documented using morphological and histological criteria, which were not sufficient to determine if these glands contained epithelial cells with changes in gene expression correlated with transformed cells (4). Accordingly, in the present studies we have characterized the epithelial cells in the mammary glands of PR-A transgenics and report that they contain populations of epithelial cells with distinct alterations in gene expression and growth potential.

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; CDK, cyclin-dependent kinase; ERα, estrogen receptor alpha; ERKO, estrogen receptor null mutant; PCNA, polymorphic cell nuclear antigen; PR, progesterone receptor; RT–PCR, reverse transcription–polymerase chain reaction.

Materials and methods

Animal treatment and tissue collection

Nulliparous adult FVB mice (10–14 weeks) were used in these studies. PR-A transgenic and PR-B transgenic mice have been described previously (4,5). Mammary glands of ERα null mutant (ERKO) mice were kindly provided by Dr Dennis B.Lubahn (9). The mice were housed and cared for in accordance with the NIH guide to humane use of animals in research.

For cell proliferation studies, mice were administered 160 μg/g body wt of 5-bromo-2-deoxyuridine (BrdU, Sigma, St Louis, MO) 2 h before death. For studies with mifepristone (RU486, Sigma), mice were treated with mifepristone 16 μg/g body wt daily for 4 days. For immunohistochemical analyses on paraffin sections, mammary tissues were collected, fixed in 4.7% formalin (same as 10% buffered formalin phosphate, Fisher Scientific, Pittsburgh, PA), dehydrated, embedded in paraffin and cut into 5 μm thick sections. For immunohistochemical analyses on frozen sections, mammary glands were mounted in OCT and quick-frozen in a mixture of dry ice and ethanol. Cryostat sections (5–10 μm thick) were cut and mounted onto glass slides and fixed for 2 min in methanol/acetone (1:1). For immunoblot analyses tissues were frozen in liquid nitrogen and stored at –70°C until use.

Antibodies

The antibodies used were: anti-BrdU, rat monoclonal antibody (Harlan Sera-Lab Ltd, Loughborough, UK); anti-polymorphic cell nuclear antigen (PCNA), clone PC10 (DAKO, Carpinteria, CA); anti-cyclin D1, mouse monoclonal antibody (Biocare Medical, Walnut Creek, CA); anti-ERα, mouse monoclonal antibody 6F11 (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK); anti-p21WAF1, mouse monoclonal antibodies Ab-5 and Ab-11 (Lab Vision, Fremont, CA).

Immunohistochemistry

BrdU-, cyclin D1-, p21- and ERα-positive cells were analyzed in paraffin embedded sections as described previously (10,11). All the mouse monoclonal antibodies used in these studies corresponded to IgG_1. Accordingly, in experiments using mouse monoclonal antibodies, for negative controls, the primary antibodies were substituted at equivalent concentrations with an irrelevant mouse IgG_1 (DAKO). The antigen–antibody complexes were identified using Universal DAKO LSAB2 labeled streptavidin–biotin peroxidase kit (DAKO). The sections were counterstained with Mayer's hematoxylin solution (DAKO). After counterstaining, nuclei negative for the antigen appeared purple-blue and positive nuclei appeared brown. Analyses for PCNA-positive cells were performed on frozen sections using an indirect immunofluorescence assay as described previously (12). In each experiment, mammary glands from a minimum of three mice were examined and mammary glands for each mouse were analyzed in triplicate. In each experiment, the percentage of immuno-positive cells was obtained by counting a minimum of 500 cells per gland. The differences between the various experimental groups were analyzed by means of a two-sided Student's *t*-test and were considered significant when $P < 0.05$ was obtained.

Western blot analyses

Protein extracts were prepared from mammary tissues of wild-type and PR-A transgenic mice by homogenization in lysis buffer [50 mM Tris–HCl (pH 8.0), 125 mM NaCl, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 1 mM PMSF] containing the following protease inhibitors: leupeptin, pepstatin, aprotinin, each at a final concentration of 1 μg/ml. The homogenates were sonicated, centrifuged at 110 *g* and the pellets were discarded. Protein concentrations in the supernatants (lysates) were determined by DC protein assay (Bio-Rad, Hercules, CA). Aliquots of lysates equivalent to 20 μg of protein were subjected to electrophoresis through 8–16% SDS–PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 10% non-fat powdered milk prior to treatment with the primary antibodies. Subsequently, the blots were washed and treated with appropriate secondary antibodies. The resulting antigen–antibody complexes were detected by ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK), the films were scanned and subjected to densitometric analyses using the PC version of NIH image (Scion Corporation).

cDNA synthesis and quantitative RT–PCR analysis for ERα

Total cellular RNA was extracted using Totally RNA isolation kit (Ambion, Austin, TX) according to the protocol provided by the manufacturer. For cDNA synthesis, 6 μg of total RNA, prepared as described above was treated with DNase I, to remove any contaminating genomic DNA, and then used for Reverse Transcriptase (RT) coupled cDNA synthesis using oligo-$(dT)_{15}$ primers and Superscript II (Life Technologies, Bethesda, MD). The RT reaction was performed at 42°C for 50 min, followed by heating at 70°C for 10 min. The resultant cDNA was either used immediately for quantitative RT–PCR or stored at –20°C for later use.

PCR reactions were performed using the ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA). The primers used for detection of ERα were the same as described previously (13). In preliminary studies, optimal experimental conditions were established and a standard curve was generated using serially diluted samples. The amount of transcripts in each sample was calculated from the standard curve and normalized to β-actin gene, run as an internal control.

Results

Rate of epithelial cell proliferation is augmented in mammary glands of PR-A transgenic mice and is restricted to aberrant epithelial structures

The proliferative status of the mammary epithelial cells in PR-A transgenic mice was examined by immunocytochemistry using two independent parameters, i.e. PCNA and BrdU. As expected, few BrdU- or PCNA-positive cells were detected in mammary ducts of wild-type mice (Figure 1A, C and E).

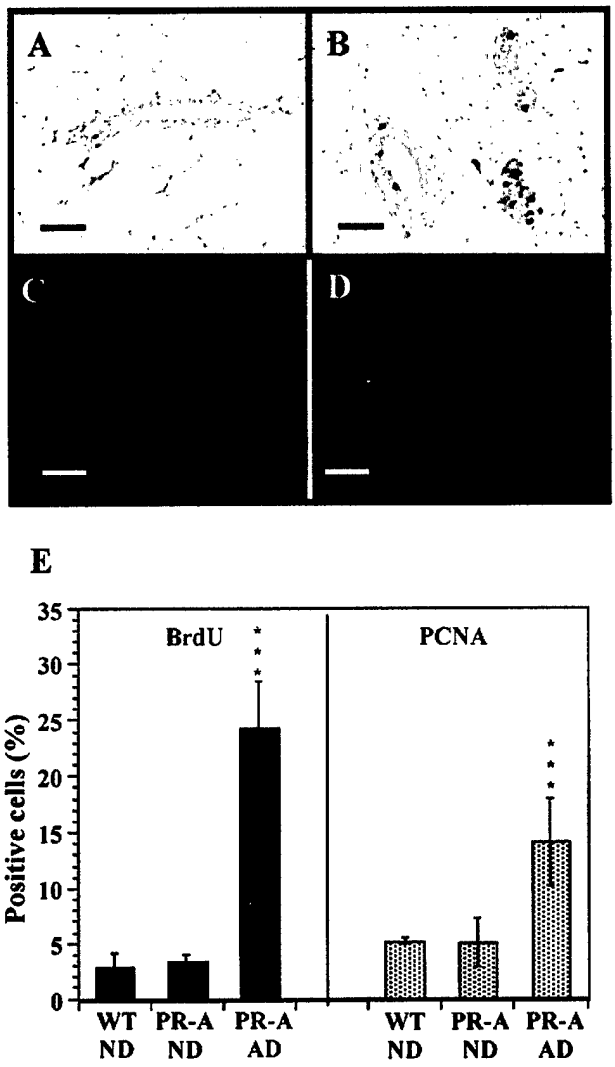

Fig. 1. Analyses for cell proliferation in mammary glands of wild-type and PR-A transgenic mice. Mammary glands from wild-type (**A** and **C**) and PR-A transgenic mice (**B** and **D**) were analyzed for immunoreactive BrdU (A and B) and immunoreactive PCNA (C and D), as described in text. Scale bar represents 20 μm. (**E**) The number of BrdU- and PCNA-positive cells in the different morphological structures were analyzed as described in text; ND: ducts with normal histology; AD: aberrant duct. ***BrdU- and PCNA-positive cells in aberrant structures are significantly higher than that in ducts with normal histology ($P < 0.001$).

Similarly, in mammary glands of PR-A transgenics, few BrdU- (3.3 ± 0.7%) or PCNA-positive cells (5.0 ± 2.2%) were detected in ducts with normal histology and were comparable with those observed in ducts of wild-type mice (BrdU: 2.9 ± 1.3%; PCNA: 5.2 ± 0.3%; Figure 1). In contrast, there was a significant increase in both BrdU- (24.3 ± 4.1%) and PCNA-positive cells (14 ± 3.9%) in aberrant mammary epithelial structures of PR-A transgenic mice (Figure 1B, D and E).

Cyclin D1 expression is elevated in the aberrant epithelium of PR-A transgenics

In mouse mammary glands, cyclin D1 is essential for mammary epithelial cell proliferation (14,15) and overexpression of cyclin D1 can lead to ductal hyperplasia (16). Therefore, we examined if the increase of mammary epithelial cell proliferation in PR-A transgenic mice was also accompanied

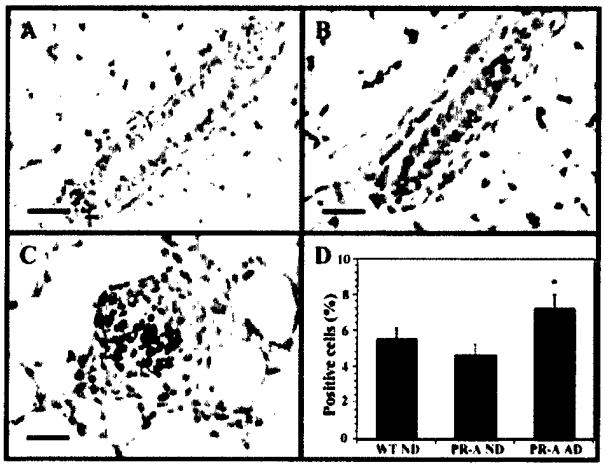

Fig. 2. Analyses for cyclin D1 expression in mammary glands of wild-type and PR-A transgenic mice. Mammary glands from wild-type (**A**) and PR-A transgenic mice (**B** and **C**) were analyzed for immunoreactive cyclin D1 as described in text. Scale bar represents 20 μm. (**D**) The number of cyclin D1-positive cells among the various morphological structures were analyzed as described in text; ND: ducts with normal histology; AD: aberrant duct. *Cyclin D1-positive cells in aberrant structures are significantly higher than that in ducts with normal histology ($P < 0.05$).

by changes in cyclin D1 expression. Immunoreactive cyclin D1 was detected in the epithelial cell nuclei of all genotype (Figure 2A–C). The number of cyclin D1-positive cells was similar between mammary ducts in wild-type mice (5.5 ± 0.6%) and ducts with normal histology in PR-A transgenic mice (4.6 ± 0.6%; Figure 2D). In contrast, there was an apparent increase in the intensity of immunostaining in the aberrant structures of PR-A transgenics (Figure 2, compare C with A and B); there was also an increase in the number of cyclin D1-positive cells (7.2 ± 0.8%; Figure 2D).

Expression of p21 is decreased in mammary epithelial cells of PR-A transgenics

Our foregoing observations on cyclin D1 expression, taken together with the patterns of immunostaining for BrdU and PCNA, strongly implied the involvement of cyclin D1 in the aberrant mammary epithelial growth. It is well known that the growth promoting effects of cyclins are achieved through their assembly with their catalytic partners, cyclin-dependent kinases, CDK4 and CDK6 and whose activities, in turn, can be constrained by CDK inhibitors (17). One of the CDK inhibitors, p21, can either act as a growth promoting or growth inhibitory factor, depending on its cellular concentration; at low concentration p21 acts as an assembly factor and thus promotes the formation of active CDK complexes while at high concentrations it inhibits CDK kinase activity (18). Consistent with this, p21 concentration has been shown to be elevated during growth suppression in human mammary tumor cells (19).

Analyses for the status of p21 expression, by immunoblot assays, revealed that it was decreased in mammary glands of PR-A transgenics (Figure 3A), which was also apparent in immunohistochemical analyses (Figure 3B). As such, while in both wild-type and PR-A transgenics, immunoreactive p21 was detected in the nuclei of epithelial cells; the overall intensity of p21 immunostaining was reduced in the ducts of PR-A transgenics as compared with the ducts of wild-type mice (Figure 3B, compare a and b). A decrease in the level

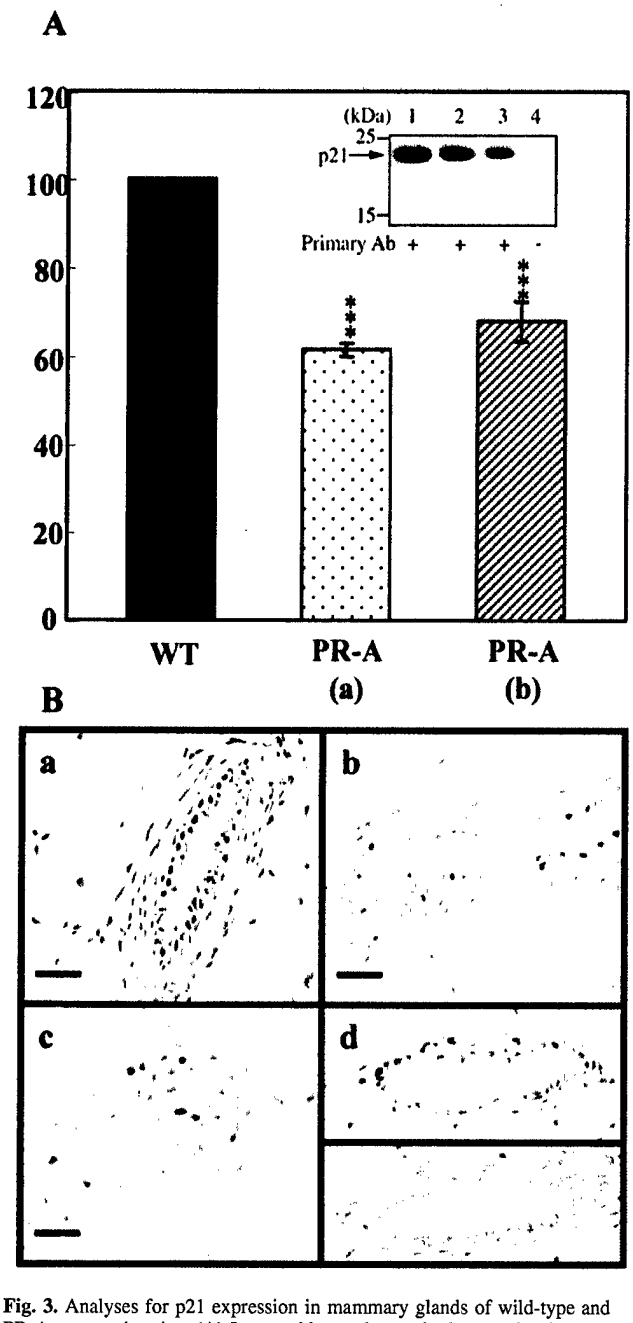

Fig. 3. Analyses for p21 expression in mammary glands of wild-type and PR-A transgenic mice. (**A**) Immunoblot analyses: the bar graphs show quantitative analyses of immunoblots by densitometry. PR-A (a) represents the data for a single sample from three separate experiments to demonstrate the low intra-sample variability; PR-A (b) shows the data corresponding to three different samples to demonstrate inter-sample variability. ***p21 expression in the mammary glands of PR-A transgenic mice are significantly lower than that in wild-type mice ($P < 0.001$). There is no significant difference between PR-A (a) and PR-A (b). The inset shows a representative immunoblot corresponding to mammary gland lysates from wild-type (lane 1) and PR-A transgenics (lanes 2–4) with (+) and without (–) treatment with the primary antibody. The position of the molecular weight standards is indicated on the left. (**B**) Immunolocalization of p21: (a) shows mammary glands of wild-type mice; (b and c) show respectively a duct with normal histology and an aberrant duct of PR-A transgenic mice; (d) shows the absence of immunoreactivity with irrelevant mouse IgG (top) and deletion of primary antibody (bottom). Scale bar represents 20 μm.

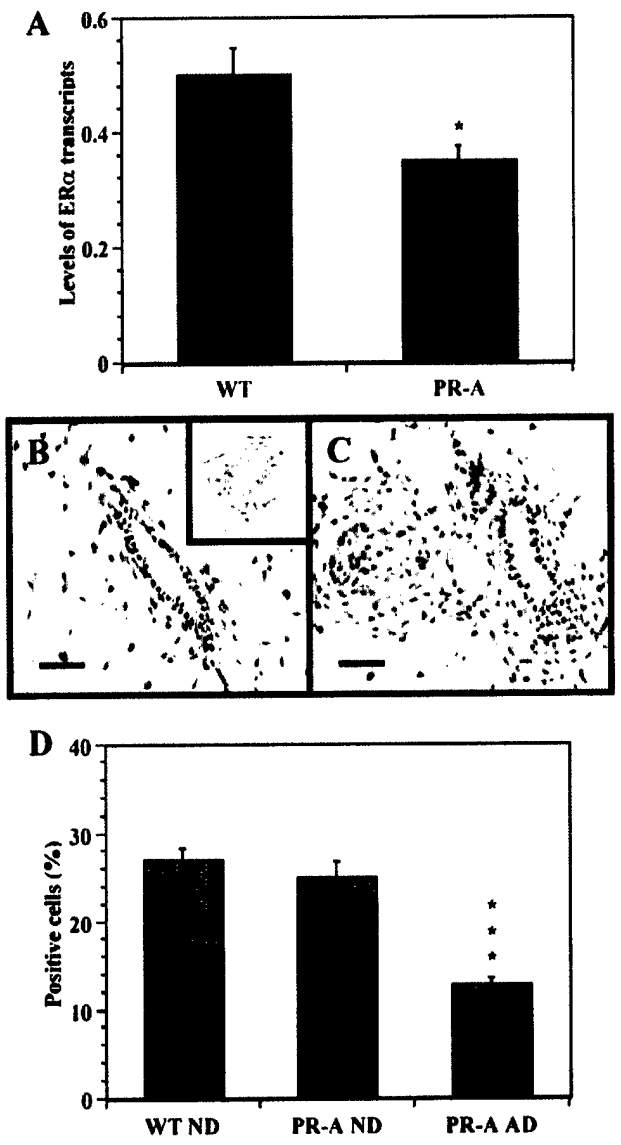

Fig. 4. Analyses of ERα expression in mammary glands of wild-type and PR-A transgenic mice. (**A**) The levels of ERα transcripts in mammary glands of wild-type and PR-A transgenic mice were analyzed by real-time RT–PCR, as described in text. The data are presented as transcript numbers (multiplied by 10^3), and represent the average ± SEM of four experiments. *The decrease in ERα transcripts in mammary glands of PR-A transgenics is significant ($P < 0.05$). Mammary glands from wild-type (**B**), ERKO (inset, B) and PR-A transgenic mice (**C**) were analyzed for immunoreactive ERα as described in text. Note that in PR-A transgenics, in the ducts with normal histology (C), the intensity of immunostaining is similar to the duct of wild-type mice shown in (B). Also note the absence of immunostaining in the ducts of ERKO mice. Scale bar represents 20 μm. (**D**) The number of ERα-positive epithelial cells in the various morphological structures were analyzed as described in text. ND: ducts with normal histology. AD: aberrant duct. ***ERα-positive epithelial cells in aberrant structures of PR-A transgenics are significantly lower than that in ducts with normal histology of wild-type and PR-A transgenic mice ($P < 0.001$).

of p21 immunoreactivity was also apparent in the aberrant epithelial structures (Figure 3B, c).

ERα expression is decreased in aberrant epithelial structures of PR-A transgenics

In a series of comprehensive studies, Medina *et al.* have identified certain molecular markers unique to mammary

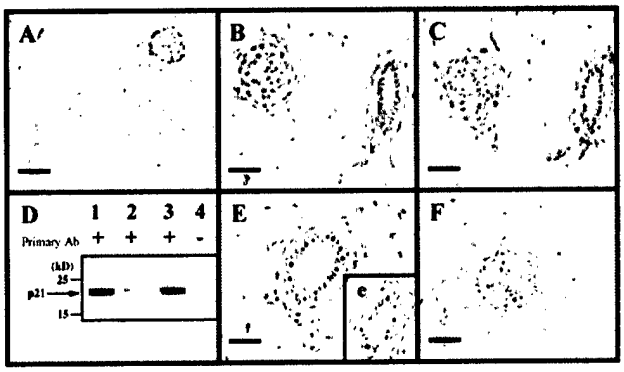

Fig. 5. Effects of mifepristone (RU486) on cell proliferation and expression of cyclin D1, ERα and p21 in mammary glands of PR-A transgenic mice. Mammary glands of PR-A transgenic mice, treated with mifepristone, were analyzed for immunoreactive BrdU (**A**), cyclin D1 (**B**), ERα (**C**) and p21 (**E**, e and **F**), as described in text. (A) BrdU-positive cells are present in the aberrant structure but not in the adjacent ducts with normal histology. (B) Cyclin D1-positive cells are present in the aberrant structure. (C) Note that the intensity of ERα immunoreactivity is less in the aberrant structure as compared to that seen in the adjacent duct. (D–F) show that mifepristone can restore the loss of p21 expression examined either by immunoblot or immunohistochemical analyses. (D) Immunoblot analyses of mammary lysates from wild-type (lanes 1 and 4) and PR-A transgenics either as is (lane 2) or treated with mifepristone (lane 3), with (+) and without (–) treatment with primary antibody. The position of the molecular weight standards is indicated on the left. (E and F) show immunohistochemical analyses for p21; note that PR-A transgenic mice treated with mifepristone; the intensity of immunostaining in both the duct with normal histology (E) and the aberrant epithelial structure (F) is similar to that seen in the duct of wild-type mice (e). Scale bar represents 20 μm.

epithelial cells in various stages of transformation. In particular, they have shown that in mouse mammary epithelial cells, a decrease in p21 expression, without an increase in cyclin D1 expression is indicative of immortalization and precedes the onset of hyperplasias/pre-neoplasias (7). Thus, from the patterns of cyclin D1 and p21 expression (shown in Figures 2 and 3), it appeared that while the ducts in PR-A transgenics had retained a normal histology they might, nevertheless, contain immortalized epithelial cells. If this were so, it would also imply that the epithelial cells in the aberrant structures contained cells in later stages of progression with increased growth potential, a characteristic of hyperplasias. Another feature that distinguishes the hyperplasias from the presumptive immortalized cells is a decrease in the expression levels of ERα (7,8). Therefore, to further verify the separate identity of the epithelial cells in the aberrant structures from those in the ducts with normal histology, we examined the status of ERα. As shown in Figure 4A, the level of ERα transcripts, analyzed by quantitative real-time RT–PCR, was significantly decreased in the mammary glands of PR-A transgenics. In immunochemical analyses both the intensity of staining and the number of ERα-positive cells in the ducts with normal histology of PR-A transgenics (24.9 ± 1.8%) were comparable with that seen in the ducts of wild-type mice (26.9 ± 1.4%) (Figure 4, compare B and C and D). In contrast, there was a decrease in both the intensity and number of ERα-positive cells (12.7 ± 0.8%) in the aberrant epithelial structures (Figure 4C and D). Figure 4 also shows the absence of ERα immunostaining in mammary glands of ERKO mice.

Table I. Number of BrdU, PCNA, cyclin D1 and ERα-positive cells in mammary glands of wild-type and PR-A transgenic mice[a]

Mammary morphology	Mifepristone treatment	BrdU	PCNA	Cyclin D1	ERα
Wild-type					
Ducts	–	2.9 ± 1.3 (143/4855)	5.2 ± 0.3 (79/1516)	5.5 ± 0.6 (200/3577)	26.9 ± 1.4 (1174/4395)
	+	0.2 ± 0.1 (9/4633)	Not done	0 (0/3699)	29.2 ± 2.9 (1057/3624)
PR-A transgenics					
Ducts with normal histology	–	3.3 ± 0.7 (160/4835)	5.0 ± 2.2 (54/1061)	4.6 ± 0.6 (160/3456)	24.9 ± 1.8 (1249/5008)
	+	0.4 ± 0.2 (14/4216)	Not done	0 (0/3531)	27.1 ± 2.3 (1205/4464)
Aberrant ducts	–	24.3 ± 4.1 (865/3563)	14.0 ± 3.9 (338/2396)	7.2 ± 0.8 (244/3392)	12.7 ± 0.8 (316/2614)
	+	26.3 ± 3.6 (639/2430)	Not done	6.6 ± 1.0 (150/2305)	14.3 ± 1.0 (275/1943)

[a]The data represent percentages of positive cells and are presented as mean ± SEM; the number of positive cells/total cells counted are presented in brackets.

Mifepristone (RU486) restores the loss of p21 in mammary epithelial cells of PR-A transgenic mice but has no effect on the expression of cyclin D1, ERα or cell proliferation

Next, we examined whether progesterone/PR signaling is involved in misregulation in cell proliferation and changes in expression levels of p21, cyclin D1 and ERα in the mammary glands of PR-A transgenics. To this end, we tested the effects of the anti-progestin, mifepristone on these various molecular parameters. Administration of mifepristone to intact PR-A transgenic mice had no effect on the morphology of mammary glands such that excessive ductal growth was still present (data not shown). Furthermore, in these glands, BrdU-positive cells were still detected in the aberrant epithelial structures (Figure 5A) and the number of these cells (26.3 ± 3.6%) was equivalent to those seen with untreated mice (Figure 1E, Table I). In contrast, mifepristone abolished BrdU immunostaining in the ducts with normal histology (Figure 5A) and also in the ducts of wild-type mice (Table I). Mifepristone also did not have any effect in the aberrant epithelial structures of PR-A transgenics with regard to cyclin D1 expression (Figure 5B) such that the number of cyclin D1-positive cells (6.6 ± 1.0%) was comparable with that observed in untreated mice (Figure 2D, Table I). Similarly, in the aberrant epithelial structures, a decrease in ERα expression, both with regard to intensity (Figure 5C) and the number of ERα-positive cells (14.3 ± 1.0%, Table I) was unaffected in mifepristone treated mice. In contrast to BrdU and cyclin D1 expression, mifepristone abolished the differences between mammary glands of wild-type and PR-A transgenics by restoring the loss of p21 as shown by immunoblot analyses and immunostaining for p21 (Figure 5D–F). There were no apparent differences between mammary glands of wild-type and mifepristone treated wild-type mice with regard to the expression patterns of p21 (data not shown).

Mammary epithelial cells of PR-B transgenic mice do not exhibit alterations in cell proliferation or in p21, cyclin D1 or ERα expression

The distinguishing characteristic of PR-A transgenic mice is that they carry an imbalance in the native ratio of A:B isoforms of PR and hence, an alteration in signaling through PR. Therefore, it was important to identify if the changes observed in the mammary epithelial cells of PR-A transgenics were indeed related to its mammary phenotype or simply resulted from abnormal signaling through PR, due to an overall imbalance in the ratio of the two isoforms. An imbalance in the native ratio of A:B isoforms of PR also exists in PR-B transgenic mice but the mammary phenotypes of these mice are distinct from PR-A transgenics (5). Accordingly, we also examined the mammary glands of PR-B transgenics. As shown in Figure 6, as compared with mammary ducts of wild-type mice, there was no detectable increase in the number of BrdU-positive cells or cyclin D1 expression in ducts of PR-B transgenics (Figure 6B and D). The expression of p21 was also not diminished in the ducts and, in fact, appeared to be somewhat elevated (Figure 6F).

Similarly, ERα expression was also unaffected in the mammary glands of PR-B transgenic mice (Figure 6H).

Discussion

Using morphological and histological criteria, we had documented previously that mammary development in PR-A transgenic mice was abnormal. In particular, we had demonstrated that these glands exhibited extensive ductal growth, loss in basement membrane integrity and cell–cell adhesion (4), characteristics commonly associated with transformed cells. In this report, we demonstrate that the expression patterns of p21, ERα and cyclin D1 are altered in the mammary epithelial cells of PR-A transgenics and are accompanied by a higher rate of proliferation as revealed by immunostaining for BrdU and PCNA (summarized in Table I). Mouse mammary epithelial cells exhibiting a decrease in p21 without an elevation in cyclin D1 are believed to correspond to immortalized cells with limited growth rate (7). In PR-A transgenics, ductal epithelial cells with normal histology have a decrease in p21 expression without an increase in cyclin D1 expression or cell proliferation. Mouse mammary epithelial cells are also presumed to be immortal if they can be propagated *in vivo*, through serial transplantation, beyond five to seven generations (20). We have serially transplanted tissue fragments from mammary glands of PR-A transgenics up to eight generations (data not shown). Thus, we conclude that these ducts contain the presumptive immortalized epithelial cells, indicative of early stages of transformation. Among the characteristics that distinguish the presumptive immortalized epithelial cells from those in hyperplasias/pre-neoplasias are increases in cyclin D1 and cell proliferation and a decrease in ERα expression (8), features associated with the aberrant epithelial structures of PR-A transgenics. Accordingly, we also conclude that these structures contain epithelial cells in later stages of transformation and correspond to hyperplasias. In this context, it is relevant to note that when PR-A transgenics are crossbred with transgenic mice overexpressing the unactivated form of

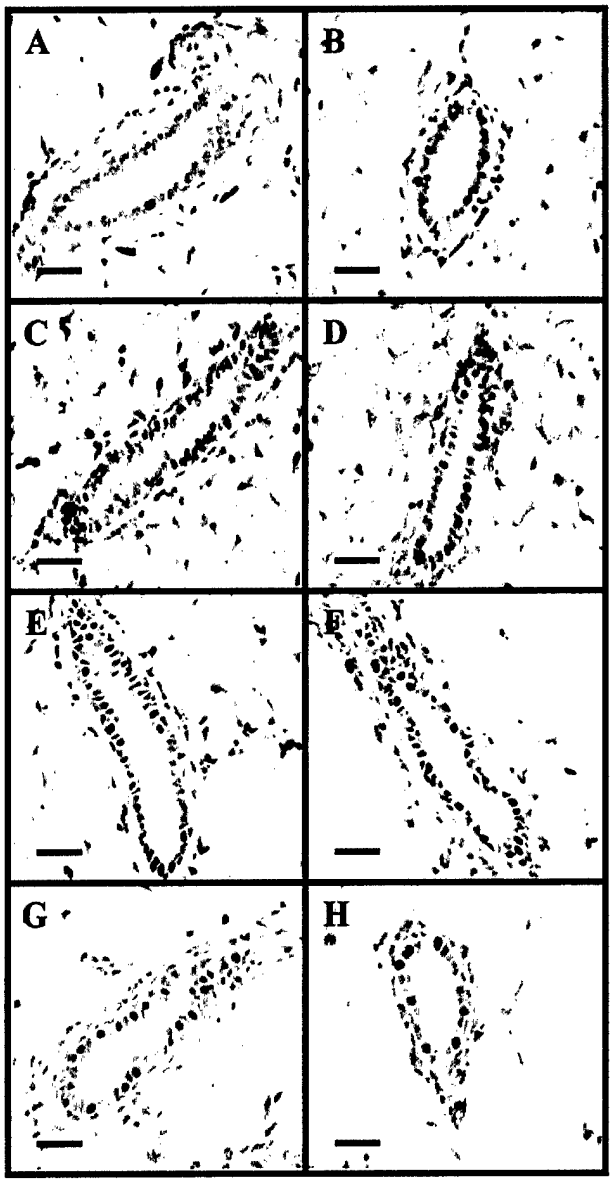

Fig. 6. Analyses for cell proliferation, cyclin D1, p21 and ERα in mammary glands of PR-B transgenic mice. Mammary glands from wild-type (**A**, **C**, **E** and **G**) and PR-B transgenic mice (**B**, **D**, **F** and **H**) were analyzed for immunoreactive BrdU (A and B), cyclin D1 (C and D), p21 (E and F) and ERα (G and H). Note that there are no apparent differences between wild-type and PR-B transgenic mice in the patterns of immunostaining for BrdU, cyclin D1, p21 and ERα. Scale bar represents 20 μm.

C-Neu, the hybrid mice develop mammary tumors with a shorter latency (21). These observations, taken together with our previous histological and morphological studies, therefore, establish that mammary glands of PR-A transgenics contain transformed epithelial cells. In turn, they reinforce our earlier proposal that an imbalance in the relative expression levels of A:B isoforms of PR can lead to abnormal mammary development and transformation of epithelial cells.

PR-B transgenic mice also carry an imbalance in the native ratio of A:B isoforms. We have shown previously that the mammary phenotype of PR-B transgenics is somewhat opposite to that of PR-A transgenics and, in particular that they do not exhibit excessive ductal growth (5). Our present studies show that mammary epithelial cells of PR-B transgenics do not exhibit significant changes in the expression levels of the various defined molecular markers, used for the identification of transformed cells in PR-A transgenics. Thus, our present studies also reveal that the transformation of mammary epithelial cells in PR-A transgenics is simply not the result of an imbalance in the ratio of A:B isoforms but specifically due to overexpression of PR 'A' form. In this context, it is noteworthy that an imbalance in the relative ratio of A:B isoforms of PR has also been observed in certain human mammary tumors, and this is often associated with overexpression of PR 'A' form (22–24).

Progesterone/PR signaling has been shown to regulate p21 expression indirectly through SP-1 sites on p21 promoter (25). A distinguishing feature of both the presumptive immortalized epithelial cells and those in hyperplasias is the loss in p21 expression. In PR-A transgenics, the loss in p21 expression is restored in both these cell populations with the antiprogestin, mifepristone, suggesting the involvement of progesterone/PR signaling. However, it is important to note that, in mammary epithelial cells of PR-A transgenics, changes in p21 expression levels do not appear to be tied to degree of cell proliferation. As such, the decrease in p21 expression in the presumptive immortalized epithelial cells is not accompanied by an increase in cell proliferation and conversely, when the loss in p21 is restored with mifepristone in the hyperplasias, they continue to proliferate. p21 is a multifunctional protein and as such, has been implicated in a vast array of regulatory networks (26). To this end, the significance of decreased p21 expression accompanying the immortalization of mouse mammary epithelial cells must await future studies.

In mammary glands of wild-type mice, cell proliferation is initiated at the onset of pregnancy in response to progesterone/PR signaling, which requires cyclin D1 (14,15). Similarly, long-term (21 days) administration of estradiol and progesterone to ovariectomized wild-type mice increases cell proliferation analogous to that occurring during pregnancy (27) and this is accompanied by an increase in cyclin D1 expression (28). In the hyperplasias of PR-A transgenics, similar to mammary epithelial cells of wild-type mice, the increase in cell proliferation is accompanied by elevated expression of cyclin D1 except that it occurs in the absence of pregnancy. Furthermore, in these cells both the increases in cell proliferation and cyclin D1 expression are insensitive to mifepristone. In contrast, mifepristone abolishes both the basal epithelial cell proliferation observed in the immortalized cells in the ducts of PR-A transgenics (Figure 5) and in mammary ducts of wild-type mice (Table I). This suggests that a principal trigger for the progression of immortalized cells to hyperplasias may be a derangement in progesterone/PR-dependent regulation of cyclin D1 expression resulting, in turn, in progesterone independent proliferation. In fact, it may even be that the mammary epithelial cells of hyperplasias have acquired a resistance to progesterone/PR signaling due to overexpression of cyclin D1, as found with T47-D human mammary tumor cells (29).

In summary, our studies show that mammary glands of PR-A transgenics contain distinct populations of epithelial cells in different stages of transformations, and hence, with different growth potential. In addition, they show that the altered growth potential of these epithelial cells is at least, in part, due to misregulation in progesterone action at the level of cell cycle. Thus, our present studies establish that an imbalance in the expression of the two isoforms of PR, resulting from

overexpression of PR 'A' form, can lead to transformation of mammary epithelial cells. These studies also highlight that PR-A transgenic mice can serve as an important experimental model for dissecting the mechanisms underlying ovarian steroid dependent mammary epithelial cell transformation and progression *in vivo*.

Acknowledgements

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Review

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The not-so innocent bystander: the microenvironment as a therapeutic target in cancer

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The microenvironment in which cancer arises is often regarded as a bystander to the clonal expansion and acquisition of malignant characteristics of the tumour. However, a major function of the microenvironment is to suppress cancer, and its disruption is required for the establishment of cancer. In addition, tumour cells can further distort the microenvironment to promote growth, recruit non-malignant cells that provide physiological resources, and facilitate invasion. In this review, the authors discuss the contribution of the microenvironment, i.e., the stroma and its resident vasculature, inflammatory cells, growth factors and the extracellular matrix (ECM), in the development of cancer, and focus on two components as potential therapeutic targets in breast cancer. First, the ECM, which imparts crucial signalling via integrins and other receptors, is a first-line barrier to invasion, modulates aggressive behaviour and may be manipulated to provide novel impediments to tumour growth. Second, the authors discuss the involvement of TGF-β1 as an example of one of many growth factors that can regulate ECM composition and degradation and that play complex roles in cancer. Compared to the variable routes taken by cells to become cancers, the response of tissues to cancer is relatively consistent. Therefore, controlling and eliminating cancer may be more readily achieved indirectly via the tissue microenvironment.

Keywords: basement membrane, breast cancer, extracellular matrix (ECM), microenvironment, stroma, TGF-β

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1. Introduction

Almost 13% of North American women will develop breast cancer, making it one of the most common forms of cancer [1]. In recent decades, early detection, hormonal prevention strategies, identification of genes associated with risk, and adjuvant therapy have had a major impact on the management and treatment of cancer. Notably, recent breast cancer initiatives by many funding agencies have focused research on the normal breast based on the rationale that the disease's frequently long latency and hormonal dependence are indicative of origins somehow circumscribed by the tissue biology.

The tissue microenvironment has often been considered an innocent bystander to the development of a tumour. An 'initiated' cell, which is presumed to contain genomic changes that endow it with new/altered features, requires additional factors acquired during progression to express this neoplastic potential. Characterisation of mutations and the identification of oncogenes have led to a better understanding of the proteins regulating neoplastic behaviour, which in turn have provided therapeutic targets for eliminating cancer [2]. However, some liken the growth of cancer to the dynamic interdependence of seed and soil – cancer occurs

when initiated cells have seeded a hospitable soil. The soil, i.e., the microenvironment, is comprised of communities of cooperating cells performing distinct functions in a complex milieu that supports and directs these activities. In multicellular organisms, cells depend on signals from their near and distant neighbours to regulate growth and function (Figure 1). There is little intrinsic 'will to live' that can be attributed to the cell *per se*; cells live or die by virtue of the presence of extrinsic survival signals [3]. Tissue pathology frequently arises from fundamental disruption of orchestrated communication between cells and among different cell types.

Perhaps the best examples are the experiments by Pierce in which carcinoma cells are induced to 'normalise' by virtue of their placement within developing embryos [4]. Despite the presence of genetic sequence alterations, these cells behave appropriately in response to the dominant influence of the microenvironment and their normal neighbours. Pierce found this to be analogous to the process of differentiation that occurs via extracellular signalling in normal tissues, and was among the first to propose that how the genome is controlled is as important as genetic change in cancer [4]. Pierce proposed that carcinogenesis is a caricature of this process, in which the regulatory controls are disrupted. Dvorak likened tumours to wounds that do not heal [5]. Indeed, for cancer to develop, it must disrupt the multitude of regulatory mechanisms by which tissues suppress abnormal growth. By understanding the response of the tissue to the presence of a cancer, new avenues appear for repressing tumour growth and malignant behaviour.

The dynamics of tumour genesis require the complicity of normal cells such as endothelial cells, inflammatory cells and the stroma [6,7]. What changes in a tissue to permit the growth of cancer? Why does it take so long; several decades in the case of breast cancer? Is the process irreversible? What are the critical molecules and mechanisms? Tumours recruit, enlist and beguile normal cells to participate in a process that is the antithesis of development. Cancer cells begin by eluding external signals from the microenvironment to establish a population, progress by thwarting suppression by normal cells and by recruiting normal cells to aberrant function, and eventually advance by destroying tissue architecture. The recognition that tumour cells depend on tissue microenvironments provides the rationale for new therapies that interrupt this recruitment.

2. Microenvironment composition

The microenvironment is composed of the extracellular matrix (ECM), soluble proteins such as growth factors, cytokines and hormones, and also encompasses the interactions between cells and between tissue compartments. Defined cell–ECM interactions are a prerequisite for the structural integrity and specialised function of breast epithelium. Whereas epithelial cells are in contact with a basement membrane (BM), stromal cells reside below, within the interstitial ECM (Figure 2). BMs are thin sheets of highly specialised ECMs present at the epithelial/mesenchymal interface of most tissues. In addition to acting physically as a selective barrier or scaffold to which cells adhere, individual components of the BM can regulate biological activities, such as growth, differentiation and cell migration, as well as influencing tissue development and repair.

BMs are biochemically complex, containing a variety of collagens, proteoglycans (PGs) and non-collagenous proteins. Some of the most abundant and well characterised proteins include laminins, entactin/nidogens, Type IV collagens and perlecan [8]. Both stromal and epithelial cells contribute to the composition of the BM. The interstitial ECM, composed of collagen Types I and III [9] and fibronectin [10], is synthesised by fibroblasts as a collagenous sheath separating epithelia from other tissue compartments.

Growth factors and cytokines are a large family of diverse soluble peptides that alter cell function by binding to specific cell surface receptors, which in turn use phosphorylation and other intracellular mechanisms to signal changes in gene expression [11]. While growth factors are predominantly produced locally, and cytokines, like interleukins, are frequently freely circulating, these classifications are not mutually exclusive and are often used interchangeably. In addition to production and receptor binding, the activity of any single cytokine depends on the context in which it is received [11]. As a result, cytokines exhibit specific bioactivities in cell culture but may have diverse, unpredictable or paradoxical effects *in vivo*. A physiological role for the ECM may be to sequester and concentrate growth factors in proximity to cell membranes since basal surfaces of epithelium express receptors for growth factors. Growth factors also affect the composition and stimulate the production of the ECM.

Information conveyed by microenvironment interactions is assimilated and integrated by cells to produce selective gene expression in a manner that is currently poorly understood. Cellular phenotypes result from the selective expression of the genome and in turn modify the microenvironment through differential production of growth factors, the ECM and other secreted products. It is clear from this brief synopsis that components of the microenvironment are critical players in conveying information necessary for function and homeostasis. The ECM in which the cell resides is both an extension of itself and a conduit for information for other cells, the organ and, ultimately, the organism. The dynamic reciprocity of this system is a key regulator of individual cell phenotype [12]. To form a tumour, cancer cells must co-opt the microenvironment superstructure; indeed, disruption of tissue architecture is a hallmark of cancer. As such, the microenvironment can also provide an important target for cancer therapy.

2.1 Role of the microenvironment in neoplasia

Cooperation among various cell types is orchestrated by incessant crosstalk via secreted proteins [13]. The differentiated state of epithelial cells cannot be maintained without appropriate

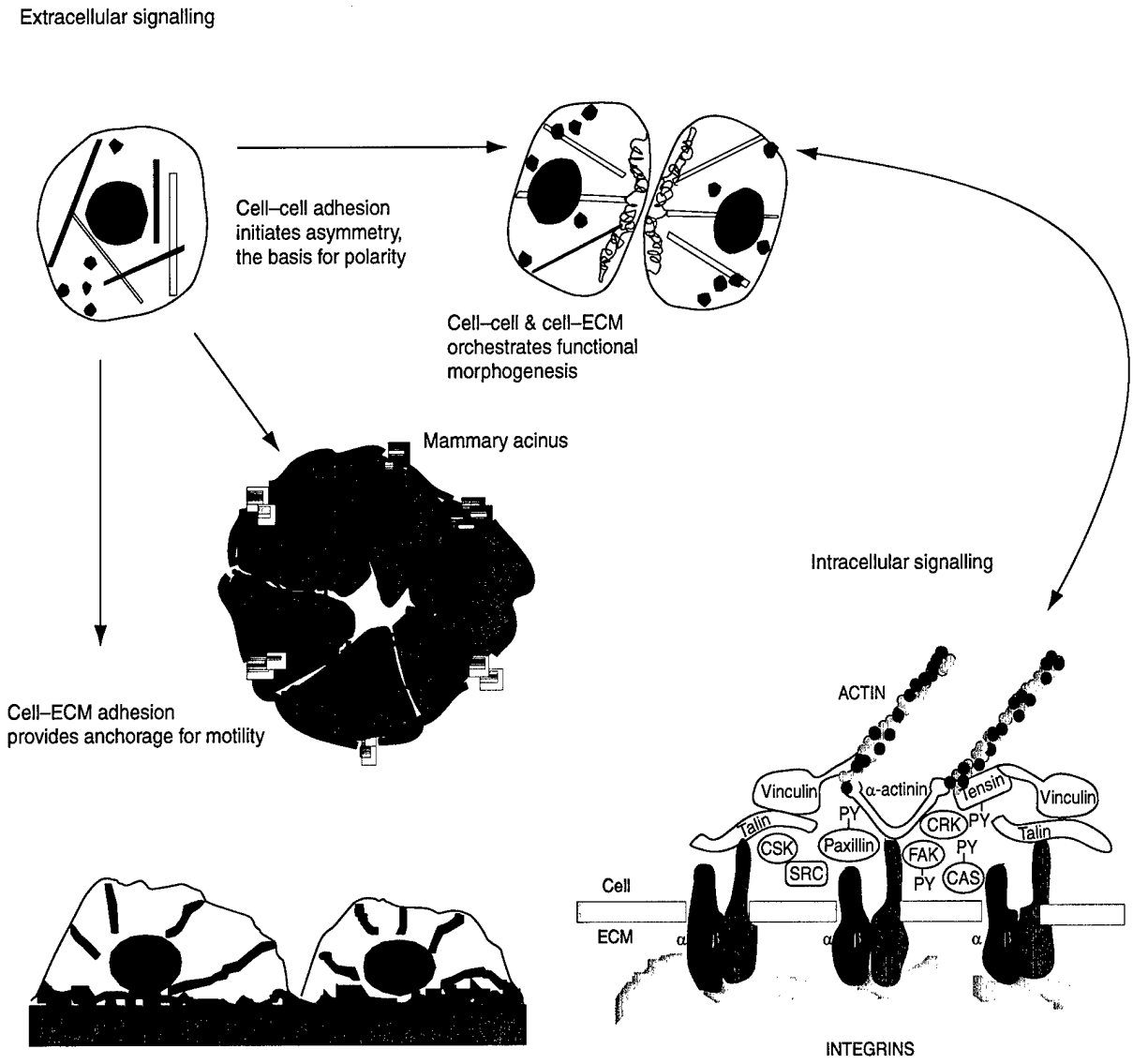

Figure 1. Cell–cell and cell–ECM interactions provide signals that allow cells to cooperate as a tissue. Signals originating from intercellular and extracellular sources are relayed from near and distant neighbours to regulate growth and function.
ECM: Extracellular matrix.

adhesion to an intact BM [14]. Normal mammary development is inhibited by blocking ECM deposition [15,16]. Conversely, tissue integrity is imperilled by processes that disrupt the flow of information between cells and their microenvironments. In general, four paradigms for the role of the microenvironment in neoplasia have been characterised based on *in vitro* and *in vivo* studies:

- Normal microenvironments suppress the expression and characteristics of neoplastic cells.
- Perturbations in the microenvironment can mediate the process of carcinogenesis.
- Changes in stromal cell contribution to the microenvironment may be conducive to the expression of preneoplasia by initiated epithelial cells or may promote progression of the preneoplastic cell.
- Microenvironment abnormalities may also result from the action of the carcinogen itself, particularly in the case of external radiation, which democratically damages all cells of a tissue.

2.1.1 *Normal microenvironments can suppress neoplastic behaviour by cancer cells*

A variety of studies suggest that expansion of an initiated population is actively opposed/suppressed by normal cells. Experimental studies show that normal tissues are capable of

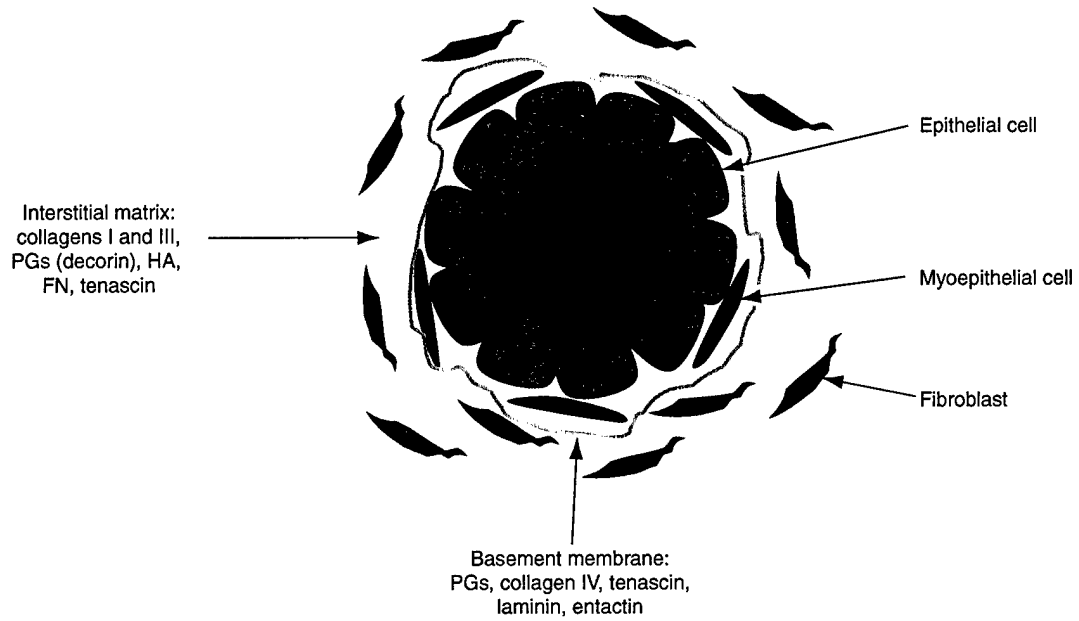

Figure 2. The mammary gland microenvironment is composed of the ECM, BM, soluble proteins such as growth factors and hormones and also encompasses the interactions between cells and between tissue compartments. The mammary epithelium consists of a single layer of luminal epithelial cells in contact with a discontinuous myoepithelium, both of which are in contact with the BM, whereas stromal fibroblasts reside within the interstitial ECM.
BM: Basement membrane; ECM: Extracellular matrix; FN: Fibronectin; HA: Hyaluronic acid; PG: Proteoglycan.

inducing differentiation of malignant tumours, despite the presence of genetic sequence alterations within the tumour cell [4,17-19]. When myogenic tumour-forming cells are transplanted into normal muscle, they are still capable of forming large amounts of muscle [20].

Recombination of carcinomas with normal mesenchyme results in varying degrees of differentiation in cancer cells [17,21]. In breast cancer, stromal cells can exert positive effects. MCF-7 breast cancer cells co-cultured with human skin fibroblasts in a collagen gel were both less proliferative and more radiosensitive [22]. Contact with normal cells induces cell cycle withdrawal and terminal differentiation of potentially malignant keratinocytes, which supports the view that normal tissue architecture acts as a dominant suppressor of early neoplastic progression [23].

Transformation in cultured cells has also provided evidence that normal cells influence the expression of the transformed phenotype. Simply altering the culture density profoundly influences the frequency with which transformed cells are morphologically evident: increased density resulted in a decreased number of transformed foci [24]. At high density, normal cells produce growth inhibitors that act in a paracrine and possibly juxtacrine manner to influence transformed cells. This phenomenon likely reflects the dynamic nature of tumorigenesis and the importance of selection in the process [25].

An intriguing and novel concept developed by Bauer states that normal cells eliminate transformed cells in culture via the induction of a short-lived soluble apoptotic signal [26]. This mechanism is readily exhibited in cultured cells, transformed by chemical, viral and physical means, is induced by TGF-β and is mediated by production of reactive oxygen species. Bauer postulates that a critical step in the establishment of a tumour is evasion of this regulatory mechanism [26]. Conversely, those tumours that do arise are shaped by the character of the tissue microenvironment, which mediates selection of clones with mutations that lead to functional alterations that are necessary for growth *in vivo*. The growth factor dependence of malignant keratinocytes expanded *in vitro* are different from those expanded *in vivo* [27].

2.1.2 Perturbations in the microenvironment can mediate the process of carcinogenesis

A corollary of the hypothesis that the microenvironment regulates normal cell phenotype is that abnormal interactions between cells and the microenvironment promote neoplastic phenotypes [12]. Elliott and colleagues [28] demonstrated that markers of malignancy were preferentially expressed when an experimental mammary tumour was grown in the mammary stroma rather than subcutaneously, and concluded that the stroma plays a role in modulating the phenotype of malignant cells. Isografting fetal salivary mesenchyme and adult mammary

epithelium accelerates the development of mammary cancer [29]. Removing and dissociating carcinogen-treated mammary epithelium for subsequent transplantation to mammary fat pad increases the expression of epithelial dysplasia compared to intact organs [30,31]. These results demonstrate that the disruption of normal stromal/epithelial interactions enhances the expression of preneoplasia.

2.1.3 Changes in stromal cell contribution to the microenvironment may promote progression of preneoplastic cells

Transformed cells both induce BM degradation and are defective in their ability to resynthesise it [32]. This concept is further supported by the studies of Sakakura [29,33] in which transplantation of fetal, but not normal adult, fibroblasts into the adult mammary gland induced the hyperplastic growth of the normal epithelial elements and rendered the epithelium significantly more sensitive to overt neoplastic transformation by carcinogenic agents. The microenvironment induced by wounding promotes the development of certain animal tumours and has been implicated in human cancer [34]. Recent studies with transgenic models indicate that inflammatory cells are critical mediators of cancer progression [35,36]. Radial scars are an independent histological risk factor for breast cancer and have recently been shown to share similar patterns of mRNA expression for several factors involved in the formation of vascular stroma [37].

Interactions between stroma and tumour cells are dynamic and reciprocal. Fibroblasts derived from normal prostate can suppress cancer growth while those derived from prostate cancer mediate progression [38,39]. Soluble factors secreted by MCF-7 cells can induce myofibroblast differentiation [40]. Also, using MCF-7 cells, it was shown that soluble factors increased fibroblast expression of matrix metalloproteinase (MMP)-2 by paracrine stimulation, but for MMP-9 expression, tumour-derived fibroblasts require direct contact with tumour cells [41].

Schor and colleagues proposed that epigenetic and genetic alterations affecting fibroblasts may lead to abnormal stromal/epithelial interactions that contribute to the development of a carcinoma [42,43]. They described a fibroblast phenotype, characterised *in vitro* by migratory behaviour similar to fetal cells, which is displayed by fibroblasts from 50% of clinically unaffected first-degree relatives of patients with hereditary breast cancer [44]. These phenotypically fetal-like cells are also found in histologically normal tissue in cancer patients [45]. Other diseases associated with increased risk for cancer have been correlated with alterations in cultured skin fibroblast phenotypes [46,47]. These observations suggest that the presence of an abnormal stromal microenvironment may precede the emergence of a clinically recognisable malignancy. Such a genetically aberrant stroma is thought to predispose an individual to cancer by increasing the frequency at which an initiated cell proceeds to neoplasia, rather than by increasing the frequency of initiation. Alternatively, such alterations in fibroblasts may reflect a genetic trait that also affects epithelial neoplastic potential. Recent identification of frequent allelic loss in the mammary stroma in patients with breast carcinoma is consistent with the latter hypothesis [48]. One possible genetically altered pathway that could lead to stromal disturbances contributing to cancer is that involved in TGF-β signalling [49].

Misregulation of adhesive properties in diseased or genetically aberrant bone marrow stroma has been suggested to play a role in haematopoietic malignancy [50]. Conversely, the therapeutic benefit of IFN-α in chronic myeloid leukaemia has been shown to be partly due to the re-establishment of cell-adhesion signals [51,52]. Greenberger and colleagues proposed a model of indirect γ-irradiation leukaemogenesis based on co-cultures of heavily irradiated bone marrow stromal cell lines that selectively bound granulocyte macrophage colony-stimulating factor (GM-CSF) receptor-positive non-irradiated haematopoietic progenitor cells, resulting in selection of tumorigenic subclones [53].

2.1.4 Microenvironment abnormalities may also result from the action of carcinogens

Carcinogens may act not only to initiate the target epithelium but also by affecting the stroma in a manner that is conducive to tumour growth, e.g., the complete carcinogens may also act as a promoter via their effects on non-initiated cells. Hodges and colleagues observed that carcinogen-treated stroma recombined with normal bladder epithelium produces neoplastic changes in epithelial morphology [54]. Research from Zarbl showed *in vivo* that mammary tumours with *Hras-1* gene mutations from N-nitroso-*N*-methylurea-treated rats arose from cells with pre-existing *Hras-1* mutations that occur during early development [55]. Thus, although clearly mutagenic in its own right, N-nitroso-*N*-methylurea exposure led to the expansion and neoplastic progression of *Hras-1*-mutation-containing populations. Similarly, continuous exposure to ultraviolet radiation not only generates additional p53 mutations in skin stem cells but preferentially promotes their expansion [56].

The authors have studied ionising radiation, a known human breast carcinogen, from the perspective of the role played by the irradiated mammary stroma. Radiation is a 'democratic' carcinogen, in that the physical event of energy deposition is independent of cell type, although the resulting biological responses are very much cell type-specific. Preneoplastic mammary cells transplanted to the mammary stroma of irradiated hosts formed tumours at high frequency only in the context of the stromal perturbations induced by radiation [57]. Similarly, a myogenic cell line forms tumours more rapidly in irradiated than in non-irradiated host muscle [20]. Production of MMP-3 (formerly referred to as transin, which is a protease that degrades BMs) by radiation-induced benign skin papillomas correlates with their high rate of conversion to malignancy as compared to chemically-induced tumours [58]. Radiation effects on stroma are typically manifested by changes in ECM composition, as evidenced by fibrosis, a well characterised result of high-dose or therapeutic radiation

exposure. Fibroblasts derived from explants of radiation-induced fibrotic skin exhibit persistent phenotypic alterations that are not seen in fibroblasts from normal wound fibrosis [59,60]. Such observations suggest that heritable changes occur in stroma as a result of radiation exposure.

If the microenvironment induced by carcinogens can shape the features (selection) and frequency (conducive) of neoplastic phenotypes, then the carcinogen 'fingerprint' may be envisioned as being built by first laying a foundation of genotypic alterations that expand in the context of a microenvironment that is the result of carcinogen-induced phenotypic change [57,61]. The authors have proposed that understanding this aspect of carcinogenesis is important since certain microenvironment alterations might be amenable to modulation, which in turn could provide the means to modify cancer progression.

Carcinogen-induced microenvironments are not necessarily mutagenic or mitogenic *per se* [61]. Rather, changes in the microenvironment may promote neoplastic behaviour by disrupting normal cell functions that are regulated through cell–cell contact, cell–ECM interactions and growth factor signalling. Thus, if ionising radiation induces a microenvironment that modifies restrictive interactions, then it may promote malignant phenotype in a manner that is functionally equivalent to the acquisition of additional mutations in the initiated cell. Alternatively, the microenvironment elicited by carcinogen exposure could create novel selective pressures that would affect the features of a developing tumour. Disruption of solid tissue interactions is a newly recognised activity of radiation as a carcinogen and a novel avenue by which to explore new strategies for intervening in the neoplastic process.

3. The mammary microenvironment

3D cell culture models of mammary breast cancer demonstrate the critical role of microenvironment interactions. Normal mammary cells grown within a reconstituted ECM form polarised acinar structures similar to those found *in vivo* and exhibit context-specific growth control [14,62]. Likewise, cancer cells act like cancer: their growth is unregulated and disorganised, which allows cancer cells to be readily distinguished from non-malignant cells [63].

Bissell and colleagues have demonstrated the power of using this information to gain insight into breast cancer. In comparing non-malignant breast cells to cancer cells cultured within the matrix, they noted an altered ratio of certain ECM receptors, called integrins. Exposing tumour cells to inhibitors of the ECM adhesion molecule, β1-integrin, caused a striking morphological and functional reversion [64]. Treated tumour cells re-establish normal acinar structures, assemble a BM, stop growing and form fewer tumours *in vivo*. Likewise, treating non-malignant cells with integrin function-altering antibodies, causes them to respond in a diametrically-opposed manner: the cells form disorganised colonies and maintain proliferation. These phenotypic reversions are reversible and are not accompanied by genomic alterations. The model illustrates that the ECM and its receptors dictate human epithelial cell behaviour, even the presence of extreme genomic alterations. The idea that restoration of appropriate cell interactions with the microenvironment can control cells with genomic alterations suggests that manipulation of the outside of the cancer cell is another route to cancer control [64] (**Figure 3**).

Changes in tumour microenvironment may act as a promoter of carcinogenesis since this ECM plays a pivotal role in restraining the spread of neoplastic cells whereas an abnormal ECM can foster invasive growth. The BM is deposited in carcinomas *in situ*, although areas of discontinuity have been described, whereas the BM is lost in invasive carcinomas [65]. Increased degradation of the BM by transformed cells is exacerbated by defective ability to resynthesise the membrane [32]. Chemically or genetically engineered disruption of the ECM in mammary glands is conducive to the expression and progression of mammary tumours [66,67]. In recent years, specific molecules of the microenvironment have been characterised as mediators of cell and tumour biology. Here, the authors discuss the role of the microenvironment in the development of cancer and focus on some ECM components as well as TGF-β1.

3.1 Fibronectin

Fibronectins (FNs) are multifunctional, adhesive glycoproteins widely distributed in connective tissue, subendothelial matrices and the stroma, as well as in many cell types. The ability of FNs to act as excellent substrates for cell adhesion and spreading promotes their involvement in cell migration during embryonic development, wound healing and tumour progression [10]. Interestingly, the loss of FN in transformed cells was the original observation that led to its discovery and characterisation. FN interacts with many other matrix components as well as several integrins [68] and syndecans-1 and -4 [69].

All FNs originate from a primary transcript, which can be alternatively spliced into three distinct regions; extradomain A (EDA), extradomain B (EDB) and Type III connecting strand (IIICS), which generates the potential of 20 different FN variants [10]. Further complexity of FN arises via post-translational modifications, such as degree of glycosylation or phosphorylation [10]. Many lines of evidence indicate that alternative splicing of FN premRNA is regulated in a cell-, tissue- and development-specific manner but is deregulated in malignancies. Loss of cell surface FN accompanies oncogenic transformation and it has been correlated with metastatic potential of breast cancer [70,71].

There is abundant evidence to support the potential therapeutic use of FN. Re-appearance of FN expression, elicited by either FN cDNA transfection [70] or signalling activation [72], has been shown to revert tumorigenic and/or metastatic phenotypes. Ruoslahti and colleagues have developed a polymeric fibrillar form of FN, sFN, by combining soluble FN with a 76 amino acid FN fragment from the first Type III repeat of FN, anastellin [73]. sFN is 10-fold more strongly adhesive to

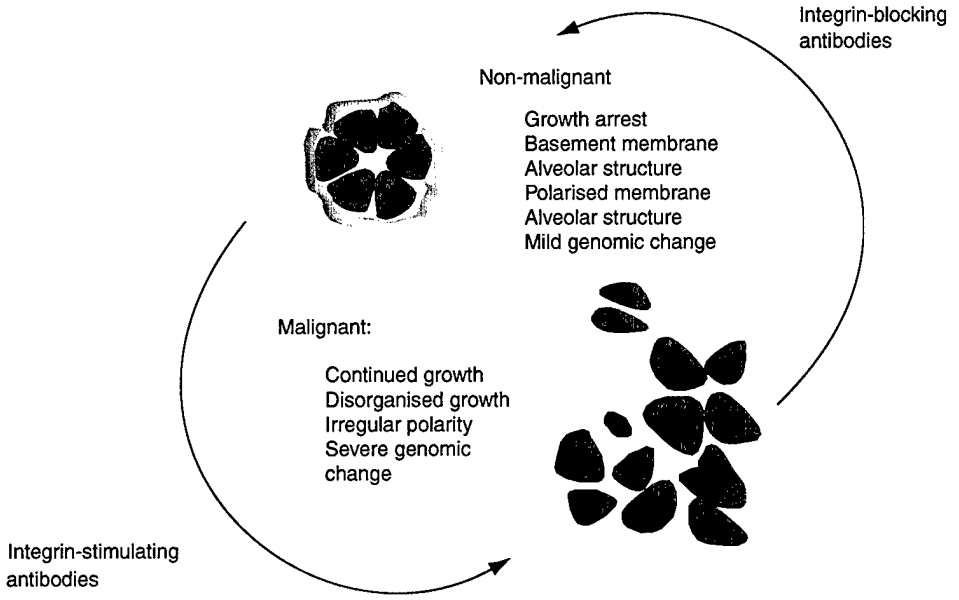

Figure 3. Non-malignant human mammary epithelial cells form acini, i.e., hollow spherical structures, when cultured in a 3D matrix, whereas malignant cells form disorganised clumps. However, when malignant cells are cultured in the presence of a β1-integrin-blocking antibody, they undergo a phenotypic reversion that resembles organisation and polarity of non-malignant cells. Likewise, disrupting integrin signalling in non-malignant cells can lead to disorganisation [64].

cells than FN coated onto plastic without polymerisation. Profound antimetastatic effects resulted when sFN was administered systemically to mice bearing various types of tumours, but no significant reduction in the growth rate of the primary tumours occurred [73]. In a later study, it was found that either anastellin or sFN could curtail both growth and metastasis of various types of xenograft tumours in mice, including tumours formed by a human breast carcinoma cell line, MDA-MB-435. The antitumour activity of these compounds is related to their ability to inhibit tumour angiogenesis [74]. Other regions of FN could also be exploited for therapeutic use; FN proteolytic fragments can suppress growth and promote apoptosis of a tumorigenic mouse mammary epithelial cell line [75].

It is possible that the splice variants of FN, especially EDA and/or EDB, could be exploited for prognosis or therapeutic use for breast cancer. Antibodies recognising EDB FN have been utilised in targeting brain tumours [76] and in nude mice bearing human tumour implants [77]. Both EDA- and EDB-containing FNs are associated with embryonic development; these isoforms are not abundant in adults tissues but reappear during wound healing and in tumour tissue ([10] references within). Immnunohistochemical studies indicate that EDA expression, in normal adult breast, is restricted to the BM region of the capillaries, with weak expression around some ducts and acini, whereas EDB is completely absent [78]. In human intraductal carcinomas, EDA expression is diffuse and moderate in the interstitial matrix, with enhanced EDA localisation at periductal rims and around tumour-containing ducts. EDA expression intensifies in invasive ductal carcinomas [78], therefore, levels of EDA FN expression are significantly higher in invasive tumours than in non-invasive ones [71]. EDB FN is expressed in the majority of breast carcinoma tissue samples regardless of histotype or grade [79]. More specifically, EDB expression has been described in intraductal carcinomas [78] as well as in invasive ductal carcinoma [80]. EDB staining was evident in the stroma near large tumour cell complexes or tumour-bearing ducts, but tumour cells lacked distinctive EDB FN. By using a combination of α-smooth muscle actin immunostaining and *in situ* hybridisation for EDB FN it was determined that the myofibroblasts of the stroma are the predominant source of EDB FN [80].

The inclusion of the EDA segment in the FN molecule is thought to result in a conformational change of the FN molecule. Consequently, the EDA FN has an increased binding affinity to integrin α5b1, rendering EDA FN twice as effective in promoting cell spreading and migration than non-EDA-containing FN [81]. Similarly, the EDB segment modulates FN conformation [82]. The α5b1 integrin is the primary FN receptor in many cell types; interaction of FN with α5b1 transduces signals that regulate cell proliferation, differentiation and apoptosis. When α5b1 is not bound to FN,

it transduces negative growth signals to the cell [81]. It is plausible that reversion of the conformational change, introduced by inclusion of the EDA or EDB segments, might suppress tumour cell growth and metastasis.

FN-induced signalling may play a role in tumour cell chemoresistance. Adriamycin-resistant MCF-7 cells are very aggressive human mammary carcinoma cells that upregulate α4b1 and α5b1 integrins compared to the wild-type MCF-7 cells, and adhere to FN via α5b1. The presence of α5b1 on the resistant cells enables them to draw advantage from FN for both cell growth and survival [83].

3.2 Laminins

The laminin (LN) family has essential roles in structural integrity, cell adhesion and signalling [84]. Cells interact with LN through a variety of cell surface receptors, including integrins, membrane-bound PGs and glycoproteins, such as dystroglycan. LNs are a large family of heterotrimeric glycoproteins encoded by three different gene products, α, β, and γ. A trimer, consisting of an α, β and γ polypeptide, forms through ionic interactions and disulfide linkages, resulting in a large cruciform-shaped complex. The 6 α, 3 β and 3 γ chains can generate more than 12 different LN isoforms [85].

For several years, LN-1 (α1β1γ1) was thought to be the most abundant LN isoform. Discovery of the α5 LN chain revealed that it is the most widely distributed α chain in adult vertebrates [86,8] and the α1 LN chain has a more restricted distribution. Early reports from the LN field must be scrutinised carefully since a widely used monoclonal antibody, 4C7, originally thought to recognise the α1 chain, actually detects the α5 chain [87]. Määttä *et al.* [88] have helped clarify some of the above-mentioned discrepancies by analysing the LN chain distribution in various types of carcinoma and normal tissues, including breast tissue and cancer. Immunohistochemical analysis showed that acini and ductal tissue of normal breast deposit α1, α2, α3, β1, β2, β3, γ1 and γ2 LN chains. Linear and well-formed BMs are present in intraductal carcinomas and LN deposition was similar to that seen in normal breast tissue. Infiltrative ductal and lobular carcinomas lack continuous BMs and have a reduction of LN chain immunoreactivity [88-90].

LN-5 (α3β3γ2) expression has been suggested as a potential tumour suppressor in the human breast [89]. Many groups agree that an inverse correlation exists between LN-5 deposition and invasiveness in breast cancer. Immunohistochemistry analysis of invasive lobular and ductal carcinomas showed a partial or total loss of LN-5 chain deposition; benign proliferations of ductal and lobular epithelium retain α3 and γ2 positive continuous BM, therefore, LN-5 expression might be a marker of benign growth. *In situ* hybridisation studies were consistent with decreased LN-5 BM deposition, downregulation of γ2 mRNA in invasive breast carcinomas [88], as well as decreased synthesis of α3 and β3 LN mRNA [89] in invasive breast carcinoma. Furthermore, γ2 mRNA is solely produced by carcinoma cells [88]. LN-5 is a major component of the anchoring filament attaching hemidesmosomes to the BM, its loss is consistent with the decrease of hemidesmosomes associated with invasive phenotypes [90].

In situ hybridisation showed that the level of α1 LN mRNA was low or moderate in carcinomas with the strongest expression in intraductal breast carcinomas; neoplastic epithelial cells also contained α1 LN mRNA, but invasive cells of infiltrative breast carcinomas only had occasional labelling [88]. β1 LN chain mRNA was mainly synthesised by stromal cells in all tumours. Infiltrative breast tumour carcinoma cells did not contain transcripts for β1 LN, but stromal fibroblasts and vascular endothelium were strongly labelled [88]. Consistent with these reports, Gudjonsson *et al.* [91] report that tumour-associated myoepithelial cells express little or no LN-1 (α1β1γ1), implying that there is a strong correlation between loss of LN-1 and breast cancer.

Augmented matrix proteolysis is believed to account for the lack of BM around invasive carcinomas. Increased serum levels of soluble LN fragments have been observed in patients with a variety of cancers. LN serum levels increase with the metastatic progression in breast cancer patients [92]. LN might promote degradation of the BM since an α1 LN chain synthetic peptide, IKVAV, mimics some of the activities of LN in promoting tumour cell adhesion, migration and gelatinase production *in vitro* and increases lung colonisation and lung metastasis *in vivo* [93]. Conversely, other regions of the LN molecule may have antitumorigenic properties. The β1 chain YIGSR peptide promotes tumour cell adhesion and migration *in vitro* but has been found to inhibit experimental metastasis in mice [94].

Ardini *et al.* suggest a possible mechanism by which the YIGSR peptide inhibits tumour cell adhesion and migration [95]. The YIGSR peptide corresponds to the LN-1 binding site for the 67 kDa LN receptor (67LR). When LN-1 binds to the 67LR, a conformation change is induced [96], which reveals a cryptic site for cathepsin B cleavage of LN-1 [95]. The fragment generated by the cathepsin B cleavage was particularly active in *in vitro* cell migration assays with MDA-MB-231 breast carcinoma cells. Release of this fragment is blocked by the addition of YIGSR peptide, perhaps because treatment with YIGSR inhibits the allosteric modification of LN structure produced by 67LR–LN interaction [95]. YIGSR has also been shown to induce apoptosis in a human cancer cell line [97]. With its many functions, the YIGSR peptide might prove to be of therapeutic benefit in breast cancer as well as in other invasive cancers.

3.3 Proteoglycans

Proteolgycans consist of a core protein with one or more covalently-bound glycosaminoglycan (GAG) chains comprised of chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) or heparan sulfate (HS). Complexity of these molecules arises from both the GAG chain, varying in length

and sulfation, as well as the individual core proteins. Hyaluronic acid (HA) is not associated with a core protein. PGs can simply be divided into two groups: cell surface PGs or matrix PGs (PGs secreted into the pericellular matrix). PGs function by regulating cell fate, controlling sequestration and diffusion of extracellular protein effectors, coordination of stromal and epithelial development and participation in cell–cell and cell–ECM interactions [98]. In addition to being modulators of growth factor activity, PGs play a fundamental role in cancer biology by forming physical and bioactive barriers to invading neoplastic cells [99].

Expression levels and localisation of many PGs in mammary tumours, as well as GAG chain fine structure, deviate from those found in normal mammary glands. Loss of differentiation and high levels of non-sulfated HA occur in tumours; HA is found in the stroma and at the surface of carcinoma cells [100]. These changes in HA are associated with the loss of differentiation and may play a role in invasion since HA seems to promote cell motility. Mammary tumours preferentially synthesise CSPG over DSPG and HSPG, and this same trend is found in cell lines derived from tumours and murine mammary-transformed cells [101,102]. It is postulated that predominance of CS in tumours weakens cell–ECM interactions thereby increasing invasive capacity of cells. As is found with HA, total sulfation of HS, isolated from mouse and human transformed and malignant cells, is grossly altered compared to their normal counterparts [98].

Cell surface PGs in mammary glands include members of the syndecan and glypican families. In human mammary tumours, syndecan-1 expression is reduced on cancer cells compared to normal cells. Induction of syndecan-1 expression, especially in infiltrating ductal carcinoma, is found on the stromal cell surface, where its expression is absent in normal breast and stromal epithelial neoplasms. This emergence of stromal syndecan-1 correlates with expression of fibroblast growth factor (FGF)-2 and tumour angiogenesis [103]. The extracellular portion of syndecan-1, ectodomain, can suppress malignant growth, stimulate actin polymerisation and induce epitheloid morphology in mouse mammary Shiongi 115 cells [104]. Furthermore, syndecan-1 has been shown to modulate wnt signalling and is critical for wnt-1-induced tumorigenesis in mouse mammary gland [105]. Glypican-1 is strongly expressed in human breast cancers, whereas its expression is low in normal breast tissue. It is thought that glypican-1 may contribute to disease progression due to the ability of breast cancer cells to exhibit a mitogenic response to multiple heparin-binding growth factors [106].

CD44 and β-glycan (known also as the Type III TGF-β receptor) [107] are also expressed in the mammary gland. These cell surface receptors are considered 'part-time' or 'facultative' PGs since they are not always glycanated. β-glycan is a possible candidate for targeting breast cancer. β-glycan levels are decreased in neoplastic human breast when compared to normal human breast tissue [108]. Ectopic expression of soluble β-glycan inhibits angiogenesis and tumour growth of MDA-MB-435 breast carcinoma cells that were inoculated into nude mice [109].

BM-associated PG core proteins have not yet been fully characterised in the normal or malignant breast. However, there is much interest in the family of small leucine-rich PGs which have been implicated principally in matrix assembly and structure, as well as in cell growth control [98]. Members of this family include lumican, biglycan, fibromodulin and decorin. Biglycan expression is low in normal breast tissue and slightly increased (correlating with the highest content of collagenous stroma) in breast tumours [110]; expression of lumican is similar [110]. Fibromodulin is not found in normal breast tissue but low levels have been detected in breast tumours [110]. Decorin protein levels are reduced in breast tumours compared to normal tissue but it can accumulate at sites of tumour invasion [110].

Recent descriptions of studies involving decorin are of great interest. Decorin can act as a powerful growth inhibitor to a wide variety of tumour cells, with diverse histogenetic backgrounds [111]; this effect is mediated by a specific interaction of decorin with the EGFR (epidermal growth factor receptor) [99]. Engineered expression of decorin had a dramatic effect on breast carcinoma cells that overexpress the potent oncogenic protein erbB2, which is elevated in 25% of breast cancers and is linked to poor prognosis. Decorin led to a 40% reduction of erbB2 expression and nearly abolished erbB2 tyrosyl phosphorylation, and that of erbB3 and erbB4 [111]. Furthermore, decorin-expressing cells failed to generate orthotopic tumours in nude mice [111]. Since its action is evocative of herceptin, modulating decorin may have therapeutic potential.

3.4 Tenascin

Tenascin (TN) is a polymorphic high molecular mass ECM glycoprotein. TN is capable of influencing cell behaviour directly through its interactions with cell surface receptors such as integrins, cell adhesion molecules of the immunoglobulin superfamily and annexin II [112]. In addition, TN can interact with other matrix molecules enabling it to play indirect roles in modulating cell behaviour [112]. *In vitro* studies with recombinant protein and TN fragments have suggested that TN is involved in cell migration [113], cell proliferation [114], promotion of angiogenesis [115] and, in some cases, acts as a cell survival factor. Although TN appears to have many functions, mice carrying a null mutation for TN have no apparent phenotype and heal normally [116].

During development, TN is highly expressed at epithelial–mesenchymal interfaces including the condensed mesenchyme of developing mammary gland. In adult murine mammary gland, TN is absent from the ductal tree [117]. It is downregulated during the development of mammary gland via interactions between the stroma and epithelium [118], and is re-expressed in mammary carcinomas [119]. Specifically, TN in normal breast is supplied by myoepithelial cells and localises to the BM, whereas both DCIS and invasive tumours show strong stromal expression

of TN [120]. Tumour TN is mainly produced by stromal cells and then recruited into the tumour tissue [120], although it has been reported that carcinoma cells may synthesise TN [121]. High expression of TN in breast carcinoma is related to poor prognosis [122].

Developmental studies indicate strict temporal and spatial control of isoform expression. Structure and size of TN varies as a result of alternative splicing of exons within its FN Type III repeats [112]. TN cell surface recognition [112] and MMP proteolytic cleavage sites are mapped to the FN Type III repeats [123]. Obviously, inclusion or exclusion of different exons generates various TN isoforms with functional diversity.

Studies concerning the differential splice variants of TN show consistent differences in the pattern of isoform expression in malignant progression. There are two commonly studied TN isoforms, a truncated isoform and an unspliced variant. Studies in breast [124], oral [125] and colorectal [126] cancers indicate that there is a switch in dominance from the truncated to the unspliced isoform. Adams *et al.* examined TN isoforms in benign, pre-invasive and invasive breast lesions [120]. Two TN isoforms, one containing exon 16 and the other containing exons 14 and 16 (14/16), were found to be associated with an invasive phenotype. The TN 14/16 isoform has also been reported in malignant ovarian tumours and some tumour cell lines.

Since TN has been implicated in cell migration, cell proliferation and promotion of angiogenesis, it was hypothesised to play a role in tumour growth and metastasis. The induction of TN expression in the tumour stroma and shift in isoforms described above further supports this idea. Surprisingly, TN was found to have a very limited role during spontaneous development and growth of mammary tumours and their metastasis to the lung. MMTV/PyV mice develop multifocal mammary adenocarcinomas early and synchronously in all mammary glands, with metastasis to the lung [127]. TN-null/MMTV/PyV mice were similar, implying that expression of TN does not influence malignant progression [128]. However, TN-null/MMTV/PyV mice had smaller tumour stroma cell nests, were surrounded by thickened cords of ECM and contained fewer monocytes and macrophages, suggesting that TN expression may influence immune surveillance and, consequently, tumour growth [128]. The role of TN in the tumour stroma is still unclear but it is perhaps more likely that TN plays an indirect role via its ability to interact with other matrix molecules. Further investigation into the functional difference of the TN splice variants will help elucidate their roles in tumour progression, and eventually a possible therapeutic target will be unmasked.

3.5 MMP and inhibitors

An important component of metastasis is the ability of cells to invade and migrate through surrounding BMs and interstitial tissue [129]. During breast cancer progression, the cells become more invasive, which is linked to increased MMP activity [129,130]. MMPs are believed to facilitate invasion and metastasis by degrading ECM components, as well as playing a role in processing growth factors. The initial step of transmigrating the BM barrier is critical, and inhibiting this will prevent both subsequent metastatic and invasive disease. This event is complex and requires the following to occur: the cells must attach to the ECM via specific receptors but at the same time secrete proteolytic enzymes that can degrade the matrix barrier; the cells must then actively migrate through the resultant BM defect. Blocking any of these events can eventually block tumour progression as shown by the recent literature [131,132].

MMPs are a family of more than 21 zinc-dependent endopeptidases, among them stromelysin-1 and -3 and the 72 kDa gelatinase A, whose expression is tightly controlled by growth factors, hormones, oncogenes and cytokines [133,134]. The majority of MMPs are produced by stromal and epithelial cells in latent proforms, i.e., lacking activity. Protease activation is an extracellular event involving proteolytic cleavage or conformational changes revealing the enzymatic site. MMP activity has been associated with cancer progression in many tumour types and contributes to tumour growth, angiogenesis, invasion and metastasis [129,135]. MMP activity can result in the production of 'matrikines', protease-generated fragments of matrix macromolecules that display cryptic bioactivities not manifested by the native, full-length form of the molecule [43].

In vitro cell invasion assays have demonstrated that ectopic expression of MMP-3 (stromelysin-1) promotes the invasive behaviour of breast epithelial cells through breakdown of the ECM as well as proteolytic cleavage of the cell–cell adhesion molecule E-cadherin [136], and ectopic expression *in vivo* promotes mammary tumorigenesis [137]. Expression of constitutively-active MMP-3 in the mammary epithelium of transgenic mice also elicits a reactive stroma, characterised by increased collagen content and vascularisation reminiscent of the stromal reaction in breast cancer [138]. These studies suggest that MMP-3 induces ECM changes that set the stage for the later development of breast tumours. Indeed, the epithelial cells from 6- to 24-month-old mice exhibited premalignant lesions and frank malignancies that exhibit various DNA losses and DNA copy number gains [137]. This transgenic model of MMP-3 activation also clearly supports a role for MMPs in cancer induction as well as progression.

Furthermore, MMP activity has been implicated in the epithelial to mesenchymal transition exhibited by aggressive tumour cells [136]. This phenotype is evidenced by a loss of epithelial cytoskeletal markers, loss of cell–cell interactions, acquisition of mesenchymal markers and pronounced invasive behaviour [139]. It is typically associated with the transition of benign epidermal papillomas to squamous cell carcinomas and increased metastatic potential [140].

Thus, MMP activity plays a central role in aberrant growth control due to disruption of regulatory controls imposed by the ECM, adhesion and cytoskeleton, and the acquisition of

not only invasive behaviour but also epithelial–mesenchymal transition [141]. Obviously, MMPs are a major class of effector molecules for remodelling the ECM and transition to metastatic behaviour in breast cancer; targeting these molecules for therapeutic use may limit the growth and spread of breast carcinoma. Inhibition of MMP synthesis, prevention of interactions between MMP and other proteins, exploitation of MMP activity and blocking MMP activity are different means presented by Egeblad and Werb [142] for anticancer therapy; the latter has received the most attention.

Under physiological conditions, tissue inhibitors of MMPs (TIMPs) inhibit MMP enzymatic activity but are not ideal candidates for therapy since some TIMPs may have cancer-promoting activities [143]. Synthetic inhibitors offer more possibilities. The first MMP inhibitor to enter clinical trials was Batimastat, which has been superseded by marimastat, an orally-active analogue of Batimastat. These inhibitors appeared to be effective in preventing metastasis of murine melanoma cells [144] and inhibiting growth of human colon cancer cells in nude mice [145]. Nude mouse xenografts of the breast cancer cell line, MDA-MD-435, developed fewer and smaller lung metastases when treated with Batimastat [146]. Phase III trials with marimastat produced dose-related side effects that included musculoskeletal toxicity, particularly tendonitis and bursitis and, disappointingly, lacked clinical efficacy in several advanced tumour states. Additional MMP inhibitors are highlighted in Egeblad and Werb [142]. Clinical trials with current MMP inhibitors have been challenging and unsuccessful. Some attribute this failure to the design of the clinical trials. Since MMP inhibitors are more likely to have a tumoristatic rather than a tumoricidal effect, a fully developed tumour microenvironment may be unaltered by treatment with a MMP inhibitor, therefore MMP inhibitors might be better suited for inhibiting early stage cancers [142,147].

4. TGF-β

Due to the scope of this review, the authors will discuss only one example (TGF-β) of the many important cytokines and growth factors in cancer to illustrate their critical and convoluted roles. TGF-β was isolated on the basis of its ability to stimulate anchorage-independent growth in rodent fibroblasts [148] but has since been shown to be a potent modulator of cellular phenotype, depending on cell type, concentration and context [149,150]. TGF-β is important in a variety of primary processes such as wound repair, inflammation, tissue morphogenesis and immune response. It elicits physiological responses at nano to picomolar concentrations, yet can be detrimental at higher concentrations. A primary mechanism controlling TGF-β activity, while making it available for rapid responses such as wounding, is its secretion as a latent complex that is sequestered in the extracellular space. Latency is conferred during protein processing by the association of TGF-β with its precursor peptide.

TGF-β1, the best studied protein of the three differentially-expressed and -regulated TGF-β mammalian isoforms, is derived from a 390 amino acid precursor. During processing, the peptide is cleaved to produce a 112 amino acid C-terminal peptide [151]. The homodimer of this peptide is noncovalently associated with a dimer of the processed N-terminal pro-segment, called the latency-associated peptide. This secreted latent TGF-β complex is unable to bind to TGF-β receptors until TGF-β is dissociated from the latent complex [152]. Physical alterations or protease degradation of latency-associated peptide releases TGF-β, which then binds to widely-distributed cell surface receptors. Thus, the biological activity of TGF-β is controlled by its release from the latent complex. This activation is considered to be the critical control mechanism for TGF-β function *in vivo*. As a result, elevated expression of the latent complex is not likely to have biological consequences, whereas increased activation, even without changes in synthesis rate, will profoundly affect physiological events [153].

Activation occurs during tissue damage, at which point TGF-β orchestrates complex tissue responses such as inflammation and repair [154,155]. TGF-β activation *in situ* was first demonstrated using an immunodetection protocol that discriminates between active and latent TGF-β [156,157]. TGF-β immunoreactivity is limited to the epithelium of murine mammary gland although latent TGF-β is highly expressed in the epithelium, fibrous stroma and adipose stroma [158]. These distinct staining patterns indicate that latent TGF-β is abundant throughout the tissue but active TGF-β is restricted to the epithelium. Likewise, in mouse skin, latent TGF-β is distributed throughout the epithelium and dermis but TGF-β is confined to the epithelium (unpublished observations). This pattern changes rapidly when tissues respond to damage. In irradiated mammary gland, TGF-β is induced while latent TGF-β is concomitantly decreased, which is indicative of activation [156]. This rapid shift of immunoreactivity also occurs in skin following phorbol ester application (unpublished observations). These data indicate that tissue damage elicits latent TGF-β activation, but activation is otherwise a restricted event [159].

TGF-β activity plays a complex role in cancer [160]. It is implicated in tumour processes that affect angiogenesis [161], reactive stroma [162,163] and immunosuppression [164,165]. As with MMPs, TGF-β activity is also associated with epithelial to mesenchymal transitions during cancer progression [166,167]. However, few studies have attempted to discriminate between active and latent but rather rely on either mRNA abundance or base protein immunoreactivity. Regardless, all studies to date indicate that TGF-β is increased in tumours versus normal tissue. TGF-β immunoreactivity correlates with breast cancer progression [168], an abnormal stroma [169] and metastases [170]. Human tumours also exhibit elevated TGF-β mRNA [171,172], immunoreactivity [163,173,174] and protein [175]. Thus, the restricted and stringently regulated activation of TGF-β found in normal tissue contrasts with the

elevated TGF-β expression observed in tumours. As a result, TGF-β has been targeted for pharmacological manipulation in cancer diagnosis and therapy [176].

In the case of breast cancer, however, studies have not yet resolved whether elevated TGF-β is prognostic for poor [168-170] or positive [171,172] outcome. Indeed, cancer therapies cause TGF-β activation that may contribute to therapeutic outcome [173,177-179]. The contradictory associations between breast cancer and TGF-β may stem from differences between detection methodologies, inability to discriminate between active and latent protein, and comparisons between localised and potentially metastatic disease [180]. Alternatively, experimental data suggest that the ability to activate TGF-β contributes to early metastatic disease [181,182]. Therefore, active TGF-β may serve as a target in advanced breast cancer [183].

5. Conclusions

Disruption of the microenvironment can take place through alterations of individual ECM components. As evidenced above, changes in protein abundance, deposition/localisation, degradation, post-translational modifications and alternative splicing of ECM components occur during tumour progression. Cytokines, such as TGF-β, cause different responses in tumour tissue compared to normal tissue, providing further evidence that the surrounding microenvironment influences neoplastic cells. Although only a limited number of molecules have been described here, it is obvious that the microenvironment should be regarded as a participant, not a bystander, in tumour progression. Further exploration into exploiting these microenvironment alterations may be fruitful for cancer therapy.

6. Expert opinion

In terms of numbers – number of cells, number of divisions, number of replicated DNA bases, number of DNA repairs and misrepairs – it is apparent that multicellular organisms must be extremely efficient in suppressing cancer. Cancer cells arise in a tissue and must overcome that tissue to be evident as clinical disease. Tremendous effort has been focused on the genetic changes that allow a cell to ignore and override external signals that direct cell function. Beginning with an emphasis on the primary control of cell proliferation, the role of oncoproteins has expanded to encompass seven acquired capabilities of cancer [2]. By turning attention to the composition of the tissue/tumour microenvironment, it is probable that additional targets will be discovered to exploit for cancer control [13,36].

Some of the targets are a function of the reaction of normal to malignant cells, for example, the induction of proteases, growth factors and ECM proteins that are a response to the wound that does not heal [5]. Since normal cells have a restricted repertoire of possible responses, it is likely that there will be common events underpinning the production of tumours that can be widely targeted. The angiogenesis inhibitors are an excellent example. This type of target may be most suitable for manipulation early in carcinogenesis or as a chemopreventative strategy.

As cancer progresses, it is clear that normal cells are recruited by the tumour and are subverted in a manner that warps phenotype, sometimes resulting in persistent phenotypic change (e.g., myofibroblasts, tumour endothelium). In this scenario, the potential lies in the juxtaposition of novel events that can lead to novel targets such as the revelation of cryptic epitopes, fetal protein forms or matrikines [43].

Finally, opportunity lies as a consequence of therapy that elicits specific changes that can then be targeted more specifically. The rapid remodelling of radiation-induced microenvironments that the authors have observed in normal mammary gland probably has counterparts in specific tumour tissues. If cancer therapy is viewed as a dynamic process in which the tumour is altered not only by cell kill but also by a changing microenvironment and tissue response following single or multiple therapies, additional features may be uncovered that are susceptible to intervention.

To exploit these possibilities, new tools must be employed. Better understanding of the effect of cancer on normal tissue (and vice versa) is required to define the windows for suppression and repression. Cancer biologists now have tools to follow simultaneous cellular features using multicolour microscopy, to monitor thousands of gene expression patterns using microarray technology and to generate mouse models that differ in the expression of specific proteins. An extensive inventory already exists of the components of cell signalling, processing and function. In order to define phenomes, which is the manner and consequence of how the genome is expressed, multiple constituent proteins, diverse cell types, cellular context and attendant morphological features should be quantitatively measured within tissues [184]. New models and methods for studying dynamic interaction of multiple cell types could provide cancer management strategies that tip the balance towards tissue suppression. Therapeutic success should include not only eradication but suppression, and pharmaceutical agents that support long-term, lifetime control of cancer as a chronic disease.

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Ionizing radiation induces heritable disruption of epithelial cell interactions

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Ionizing radiation (IR) is a known human breast carcinogen. Although the mutagenic capacity of IR is widely acknowledged as the basis for its action as a carcinogen, we and others have shown that IR can also induce growth factors and extracellular matrix remodeling. As a consequence, we have proposed that an additional factor contributing to IR carcinogenesis is the potential disruption of critical constraints that are imposed by normal cell interactions. To test this hypothesis, we asked whether IR affected the ability of nonmalignant human mammary epithelial cells (HMEC) to undergo tissue-specific morphogenesis in culture by using confocal microscopy and imaging bioinformatics. We found that irradiated single HMEC gave rise to colonies exhibiting decreased localization of E-cadherin, β-catenin, and connexin-43, proteins necessary for the establishment of polarity and communication. Severely compromised acinar organization was manifested by the majority of irradiated HMEC progeny as quantified by image analysis. Disrupted cell–cell communication, aberrant cell–extracellular matrix interactions, and loss of tissue-specific architecture observed in the daughters of irradiated HMEC are characteristic of neoplastic progression. These data point to a heritable, nonmutational mechanism whereby IR compromises cell polarity and multicellular organization.

Epidemiologic data indicate that women exposed to ionizing radiation (IR) for either therapy (1, 2), diagnostic purposes (3), or as a consequence of atomic bombs (4) have an increased risk of breast cancer. The action of IR as a DNA-damaging agent, and consequently as a mutagen, is widely considered to be the basis for its action as a carcinogen (5). However, tissue response to radiation, and hence risk, is a composite of genetic damage and epigenetic events, such as altered intercellular communicaton (6). Recent experimental models suggest that carcinogenesis can be driven by abnormal interactions between cells and their microenvironment (reviewed in refs. 7 and 8). We have shown that irradiated mammary stroma promotes tumorigenesis of unirradiated mammary epithelial cells (9), and that transforming growth factor β1 (TGF-β) activation mediates cellular and tissue response to IR (10–12). Thus, in addition to causing DNA damage, radiation exposure alters key regulators of cell phenotype that affect, directly or indirectly, the ability of normal tissue to suppress abnormal cell growth (13).

Epithelial cells depend on signals from the microenvironment to establish the requisite polarity for functional differentiation (14). Release from these constraints has profound consequences on tumorigenesis, progression, and metastasis (reviewed in refs. 6, 8, and 15). Tumorigenic and nontumorigenic human mammary epithelial cells (HMEC) are nearly indistinguishable when cultured as monolayers, but readily diverge in terms of morphogenesis in an appropriate microenvironment, which is evident in a three-dimensional reconstituted basement membrane (3D rBM) assay that we developed (16). In this assay, nonmalignant HMEC arrest growth and form lumen-containing acini similar to those found *in situ*, whereas breast cancer cells continue to proliferate and aggregate, rather than organize. Formation of acini requires expression and appropriate localization of proteins involved in the establishment of tissue structure and polarity (17). HMEC colonies that develop into phenotypically normal acini exhibit among other markers, E-cadherin at the interface between cells, basolateral β1-integrin, and basal α6-integrin (18). In contrast, breast cancer colonies exhibit disorganized, decreased, or aberrant expression of these markers, similar to what is observed in primary breast cancer.

If radiation exposure affects not only the phenotype of stromal but also of epithelial cells, such alterations could potentially promote neoplastic progression in susceptible cells. To test this hypothesis, we asked whether sublethal IR doses perturbed the ability of HMEC to undergo mammary-specific morphogenesis in a physiological context by using the 3D rBM assay. To replicate a key component of the irradiated stroma (10–12), TGF-β was added to some cultures. To measure the global consequences of irradiation, we used confocal microscopy and an imaging bioinformatics system for integrated image acquisition, annotation, and hierarchical image abstraction to register localization and expression information of targets along with positional references and morphological features (19).

We found that irradiated single HMEC gave rise to colonies where nearly all progeny failed to establish basal polarity and lost organizational integrity as measured by several parameters. As shown by quantitative image analysis, these changes were shared by the majority of the population. This finding is inconsistent with a radiation-induced mutational mechanism, which was confirmed by the absence of measurable changes in the population genome analyzed by comparative genomic hybridization analysis. Moreover, because the phenotype is exhibited by the daughters of individually irradiated cells, these data suggest that radiation causes a heritable alteration in pathways affecting cell adhesion, extracellular matrix (ECM) interactions, epithelial polarity, and cell–cell communication. Thus, epigenetic events after radiation exposure disrupt multicellular organization, which we postulate will override the positive influence of tissue architecture that usually impedes neoplastic progression.

Methods

Cell Culture. HMT-3522-S1 human mammary epithelial cells (S1; passages 53–60) were grown as described (18). Although phenotypically normal and nonmalignant, the S1 are an established cell line that have a number of chromosomal changes and an extended life span in culture (20). S1 cell monolayers were grown until 70% confluent before trypsinization, and single cells (8×10^5 cells per ml) were embedded into Matrigel (Collaborative Research) with or without 400 pg/ml recombinant human TGFβ1 (R & D Systems) and irradiated within 5 h by using ^{60}Co

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Abbreviations: IR, ionizing radiation; TGF-β, transforming growth factor β1; ECM, extracellular matrix; HMEC, human mammary epithelial cells; 3D rBM, 3D reconstituted basement membrane.

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γ-radiation at a dose rate of 90 cGy/min to a total dose of 2 Gy. Dosimetry was determined by using a Victoreen ionization chamber. Control plates were sham irradiated. Media were changed on alternate days. Cells were grown in the presence of epidermal growth factor for 6 days, and harvested at 10 days. For immunocytochemistry cultures were embedded in Tissue-Tek compound (Sakura Finetek, Torrance, CA), and frozen in a dry ice/ethanol bath. Blocks were stored frozen until time of sectioning.

Immunofluorescence. Cryosections (20 μm) were cut at −30°C onto gelatin-coated coverslips. Sections were fixed by using methanol/acetone (1:3) at −20°C for 10 min or 4% paraformaldehyde for E-cadherin. Nonspecific sites were blocked by using the supernatant from a 0.5% casein/PBS (pH 7.4) solution for 1 h at room temperature (RT). Sections were incubated in primary antibody diluted in blocking buffer for 1 h at RT in a humidified chamber. Antibodies used were rat anti-mouse CD29 (Pharmingen) to integrin β1 chain monoclonal antibody, rat anti-human CD49f monoclonal antibody (Pharmingen) to integrin α6 chain, and mouse monoclonal antibody to E-cadherin (BD Transduction Laboratories). Sections were washed in PBS containing 0.1% BSA, before incubating in secondary antibody conjugated to Alexa Fluor 488 (Molecular Probes) for 1 h at RT in a dark humidified chamber, washed, and counterstained with TO-PRO-3 iodide (Molecular Probes), before mounting with Vectasheild mounting medium (Vector Laboratories, Burlingame, CA).

Image Acquisition, Processing, and Analysis. Dual immunofluorescence confocal images were acquired by using a Zeiss LSM 410 inverted laser scanning confocal microscope equipped with an external argon/krypton laser. Confocal images were captured at 0.5-μm intervals as 8-bit images by using a Zeiss Fluor ×40 (1.3 numerical aperture) objective. Images were standardized by comparing only images stained with the same antibodies in the same experiment, captured with the same parameters at the same times, and scaled and displayed identically. Relative intensity of images was scaled by using SCILIMAGE (TNO Institute of Applied Physics, Delft, The Netherlands), which was used to define a standard sized region of the TO-PRO-3 iodide image (nuclei slice) without reference to the Alexa Fluor 488 images. Statistical significance of the mean fluorescence intensity for each region of interest ($n = 20$ colonies) and standard error for each treatment group was determined by using the unpaired Student's t test (PRISM, GraphPad, San Diego). The displayed images were those closest to the mean intensity for the treatment group.

Segmentation of nuclei was used to determine acinar organization at the colony midsection (21). This model-based approach assumes that the projection of each nucleus is quadratic in the image space. Instead of grouping step and roof edges, the segmentation is initiated from a representation that corresponds to the zero crossings of the image. The zero crossing image is then filtered with geometrical and illumination constraints to form binarized clump of nuclei, which is then partitioned into several nuclei through a process that is called centroid transform.

Protein Extraction and Immunoblotting. Cells in the 3D rBM assay were isolated by ice-cold PBS/EDTA (0.01 M sodium phosphate, pH 7.2, containing 138 mM sodium chloride and 5 mM EDTA) (18) and lysed in buffer as described (18). Equal amounts of protein lysates were run on reducing SDS/PAGE and then immunoblotted and detected by using a Pierce SuperSignal system (Pierce). Blots were also probed for β-actin to assess equal loading of protein. Exposed films were scanned and subjected to densitometric analysis for the determination of relative amount.

Comparative Genomic Hybridization. Array comparative genomic hybridization was performed at the University of California, San Francisco, Cancer Center as described (22). Briefly, 1 μg each of test and reference genomic DNAs were fragmented by DPNII digestion, labeled by random priming with CY3- and CY5-dUTP (Amersham Pharmacia), respectively, coprecipitated with 80 μg of human cot-1 DNA (Life Technologies), and resuspended in 20 μl of hybridization buffer (50% formamide/10% dextran sulfate/2× SSC/2% SDS/200 g of yeast tRNA). This mixture was denatured at 75°C for 10 min followed by 60 min at 37°C. Just before hybridization, array slides were processed following the manufacturer's recommendations (Surmodics, Eden Prairie, MN). A frameseal frame was placed around each array, hybridization mix was added, and the slide was placed in a plastic slide holder, prewarmed to 37°C, containing 200 μl of wash buffer (50% formamide/2× SSC) to prevent evaporation. Hybridization was carried out at 37°C for 48–72 h on a gently rocking platform. After hybridization, slides were immersed for 15 min at 48°C in wash buffer, followed by washes at 48°C in 2× SSC, 0.1% SDS for 30 min, and 0.1 M sodium phosphate buffer containing 0.1% Nonidet P-40, pH 8.0, at RT for 10 min. Slides were then rinsed in 2× SSC and dried by centrifugation.

Results

Progeny of Irradiated Cells Exhibit Perturbed Cell–ECM and Cell–Cell Adhesion. To determine whether IR alters the ability of epithelial cells to functionally interact with the microenvironment, we used the 3D rBM assay of morphogenesis in a laminin-rich basement membrane where changes in tissue structure can be quantified (16). Single HMT-3522 S1 human mammary epithelial cells were cultured, with and without the addition of TGF-β, and irradiated with a dose of 2 Gy, except where noted, 3–5 h after plating in Matrigel. Surviving cells, ≈80% (not shown), formed multicellular colonies over 5–7 days and then underwent morphogenesis into hollow spheres that recapitulate mammary acini by day 10. Immunofluorescence of β1 and α6 integrins and β-catenin at the colony mid-section were analyzed by using confocal microscopy (Fig. 1*A*). HMEC colonies express basolateral β1-integrin and basal α6-integrin, which are critical for acinar organization (23). HMEC colonies arising from irradiated cells exhibited increased β1-integrin immunoreactivity that was distributed throughout the cytoplasm (Fig. 1*A*). In contrast, the immunoreactivity of α6-integrin, which partners with β4 integrin, was decreased in colonies generated from irradiated cells. A collagen IV-containing basement membrane was observed in all treatment groups, indicating that changes in integrin expression were not caused by the lack of appropriate ligand for this ECM receptor (not shown). Treatment with TGF-β did not alter β1 integrin localization but did reduce α6 integrin immunoreactivity further. β-catenin, which is involved in cell–cell adhesion via the cytoskeleton and E-cadherin, was localized to the lateral cell borders in colonies from nonirradiated cells. β-catenin immunoreactivity was decreased in colonies derived from irradiated cells.

Disrupted Tissue-Specific Morphogenesis and the Irradiated Phenotype as Quantified by Image Analysis Reveal a Global HMEC Response. The use of morphogenesis as a readout of cellular function requires systematic analysis of colony organization and protein localization to classify the degree of response. It is therefore desirable to conduct population studies and correlate features measured from images of cells with their treatment. The acinar-like organization of colonies was analyzed by using the relative nuclear position in confocal optical midsection as described in *Methods* (Fig. 1 *B–D*). The degree of acinar organization around a central lumen was determined by fitting the nuclei to an ellipse (Fig. 1*B*). Acinar organization was significantly ($P < 0.0001$) reduced in colonies arising from irradiated cells that were

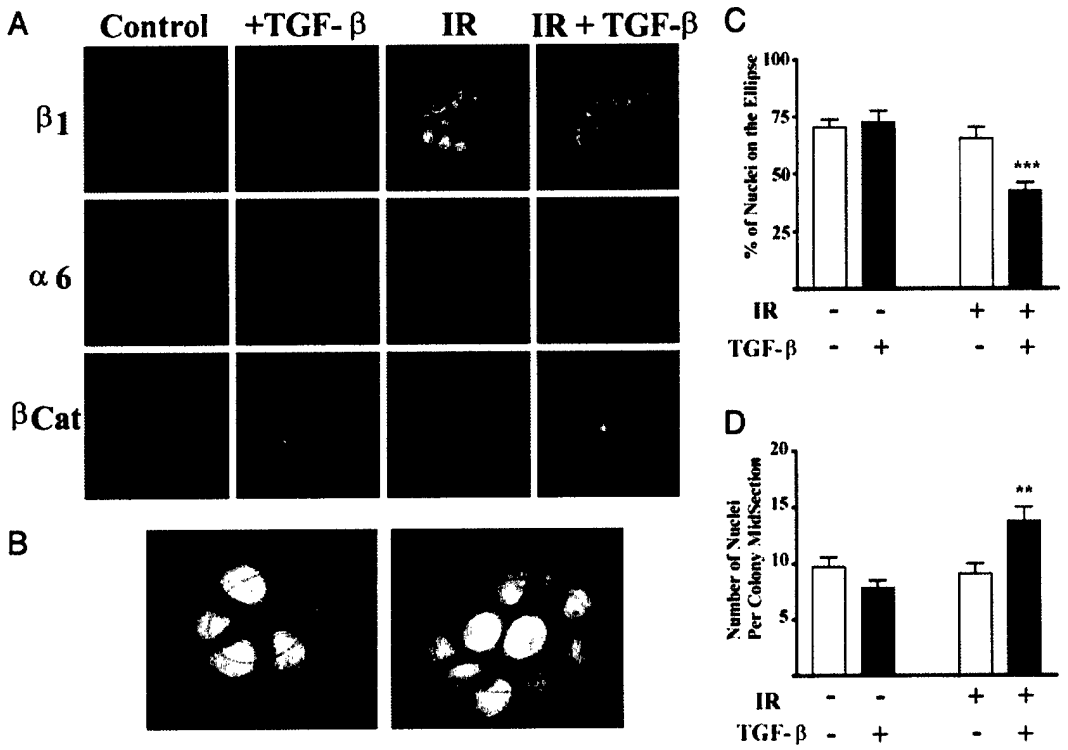

Fig. 1. Perturbed protein localization and acinar organization as a function of TGF-β, IR, and dual treatment. Colonies develop and organize in 3D rBM culture over the course of 10 days, during which time the cells are fed every other day. EGF, to stimulate proliferation, is removed at day 6, and the cells are harvested at day 10. (*A*) Representative images of colonies from control, TGF-β-, IR-, or dual-treated cultures. The image is representative of the mean intensity for each marker based on image analysis of 20 randomly chosen colonies. Immunostaining of β1 integrin, α6 integrin, and β-catenin was detected by using secondary antibodies labeled with Alexa Fluor 488 (green). Nuclei are counterstained with TO-PRO^R-3 iodide, shown in red. Note the loss of acinar organization in the irradiated colonies. (*B*) Acinar organization was measured by nuclear segmentation of the colony confocal midsection fit to an ellipse as shown for a control (*Left*) and dual-treated (*Right*) colony. (*C*) Acinar organization as a function of treatment group ($n > 100$ colonies per treatment). Acinar organization was significantly ($P < 0.0001$) decreased in colonies that arose from irradiated cells that were cultured in the presence of TGF-β. (*D*) The number of nuclei per colony midsection as a function of treatment group. The number of nuclei was significantly ($P < 0.001$) increased in colonies arising from irradiated cells treated with TGF-β.

cultured with TGF-β. The number of cells per midsection was also significantly increased ($P < 0.001$) in irradiated, TGFβ-treated HMEC colonies in comparison to colonies from control cells or those exposed to single agents.

The assembly of cells into tissue-specific structures requires the interaction of different cell adhesion systems. E-cadherin is a crucial epithelial adhesion molecule that links cells via an homophilic extracellular domain and is anchored intracellularly to the cytoskeleton via dynamic interactions with the catenins (24). Low E-cadherin immunoreactivity in breast cancer is associated with poor prognosis (25), whereas restoration of E-cadherin reverts the invasive phenotype of cancer cells (26). We localized E-cadherin by using immunofluorescence, confocal microscopy and image analysis (Fig. 2). Colonies from irradiated cells cultured in the presence of TGFβ showed a significant ($P < 0.0001$) loss of E-cadherin immunoreactivity compared with control cells. The unlikely possibility that the colonies surviving treatment were selected from a previously existing population was addressed by examining the distribution of individual colonies within each treatment group in comparison to control colonies. A representative analysis is shown for E-cadherin, indicating that the dual treated colonies form a distinct population (Fig. 2*C*). To determine whether the effect on cell interactions was sensitive to radiation dose, we performed a dose–response (Fig. 2*D*). E-cadherin immunoreactivity was significantly decreased in colonies arising from cells exposed to as little as 25 cGy, a dose that does not result in appreciable cell kill. To determine whether radiation exposure and TGF-β treatment resulted in significant changes in the genomic sequence, we performed comparative genomic hybridization as described in *Methods*. This analysis did not reveal any significant differences between the untreated and double-treated populations (data not shown), which supports the global population response revealed by quantitative image analysis.

There is an intricate relationship between cell–ECM and cell–cell adhesion in glandular tissues. To determine whether other cell–cell adhesion molecules also change, we measured connexin 43, a member of a family of proteins that assemble into gap junctions and modulate the transfer of molecules between cells. Breakdown of gap junctional complexes is induced by tumor promoters (27) and correlate with breast cancer metastatic potential (28, 29). Connexin 43 is also associated with the function and signaling of E-cadherin (30, 31). In S1 HMEC acinar colonies, connexin-43 was localized as distinct aggregates between cells. The number of connexin 43 foci per colony decreased after radiation exposure, regardless of TGF-β exposure (Fig. 3). When normalized to the number of cells per colony, the frequency of connexin foci decreased >3-fold in the daughters of irradiated cells (2.0 ± 0.46, $n = 8$) compared with those from unirradiated cells (6.9 ± 1.1, $n = 18$).

Decreased E-Cadherin and β-Catenin Localization Are Not a Function of Protein Abundance. E-cadherin localization can be modified by the degree of association with the cytoskeleton via the catenins. β-catenin and E-cadherin partner to link cells and the cytoskeleton via the adherens junction (32). To test whether decreased immunolocalization was caused by a change in the compartmentalization of these adhesion molecules, sections were detergent

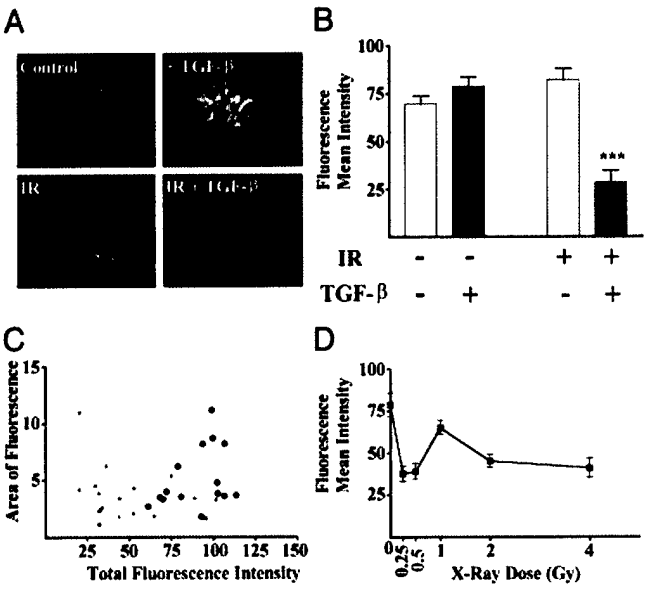

Fig. 2. E-cadherin immunoreactivity and localization are significantly reduced by IR and TGF-β. (*A*) Confocal images of E-cadherin immunoreactivity in midsections of colonies representative of the average response as measured by image analysis of 20 colonies are shown for each treatment group. E-cadherin (green) and nuclei (red) were detected as described in Fig. 1. (*B*) Quantified E-cadherin immunoreactivity as a function of treatment group. The mean intensity of E-cadherin immunofluorescence was significantly ($P < 0.0001$) reduced in TGF-β-treated, irradiated colonies. (*C*) Display of relative intensity versus colony area for sham (black circles) and dual-treated (red triangles) colonies. Comparison of the treated to control populations show that >75% of the treated colonies exhibit loss of E-cadherin, a frequency that cannot be explained by mutation rates. (*D*) Quantified E-cadherin immunoreactivity as a function of radiation exposure. The dose–response shows significant loss of E-cadherin immunoreactivity at doses that do not lead to any detectable loss of cell viability.

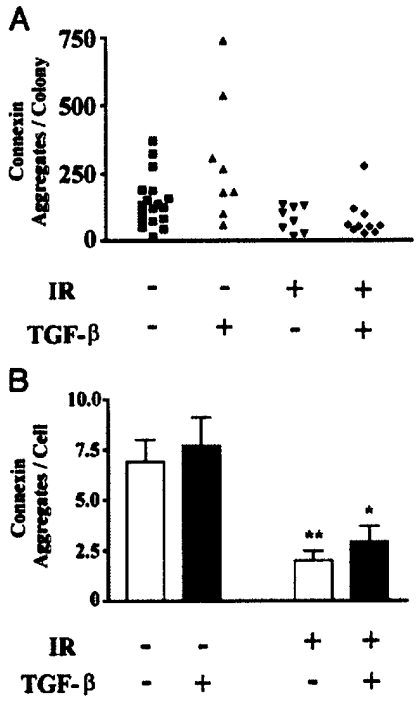

Fig. 3. Gap junctions are decreased in irradiated colonies. (*A*) Connexin 43 was localized by immunostaining and randomly selected colonies were imaged by confocal microscopy. Data shown are representative of two independent experiments. The number of aggregates per colony are displayed for 8–18 colonies per treatment. (*B*) The average (±SE) number of connexin 43 foci per cell is displayed as a function of treatment group. Colonies arising from irradiated cells showed significantly ($P < 0.05$, two-tailed *t* test) fewer connexin foci than those from nonirradiated cells.

extracted to remove the soluble fraction. Detergent extraction before fixation did not alter the pattern or intensity of E-cadherin in dual-treated samples (data not shown). Consistent with this finding, immunoblotting total protein extracts showed that both E-cadherin and β-catenin levels were decreased in TGF-β-treated colonies (Fig. 4*A*). β1 integrin protein abundance, on the other hand, increased in irradiated samples regardless of TGF-β exposure, which is consistent with the increased cytoplasmic staining shown in Fig. 1*B*. In contrast, only the progeny of irradiated cells showed a decrease in E-cadherin immunolocalization. These data suggest that decreased E-cadherin immunoreactivity at the cell junctions in the dual treated colonies reflects both a TGF-β-induced decrease in protein levels and a radiation-induced change in localization, suggesting a change in complex formation at the cell surface.

Together, these data indicate that IR can generate a persistent phenotype in daughter cells characterized by increased cytoplasmic β1 integrin, decreased α6 integrin, radically decreased cell surface localization of E-cadherin and β-catenin, and loss of connexin 43. The cumulative epigenetic changes in phenotype results in a loss of tissue-specific architecture that is indicative of malignant progression.

Discussion

In this study we show that irradiated single HMEC gave rise to colonies that had more cells, failed to establish tissue-specific organization, and expressed significantly less E-cadherin, β-catenin, and connexin-43. It is remarkable that the phenotype was exhibited by progeny of individually irradiated cells, suggesting that IR causes heritable alterations in pathways affecting cell adhesion, ECM interactions, epithelial polarity, and cell–cell communication. Release from cell–cell interactions, as demonstrated by experimentally induced loss and restitution of E-

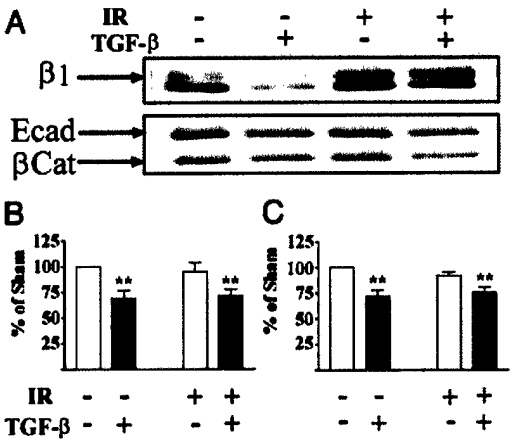

Fig. 4. Protein levels as a function of IR and TGF-β. (*A*) Representative immunoblots of β1 integrin, E-cadherin, and β-catenin from total protein extracted from cultures. β1 integrin protein abundance increased in irradiated samples, regardless of TGF-β exposure. E-cadherin and β-catenin protein abundance were decreased in extracts from TGF-β-treated cultures, regardless of irradiation. Quantitation of E-cadherin (*B*) and β-catenin (*C*) protein abundance from three independent experiments normalized to β-actin are shown as mean and standard error. The protein levels in cell extracts were significantly ($P < 0.01$) reduced in TGF-β-treated cultures.

cadherin (26, 33), has profound consequences for breast cancer tumorigenesis, progression, and metastasis. The features of individual colonies measured by quantitative image analysis showed that these changes were present in the majority of the population, a finding inconsistent with the frequency of radiation-induced mutations and confirmed by the absence of measurable changes in the population genome. Thus epigenetic mechanisms initiated by irradiation of HMEC result in a malignant-like phenotype in progeny generations after IR exposure.

Intercellular and extracellular signals are critical to the suppression of neoplastic cellular behavior. Disruption of cell–cell interactions are implicated, if not required, in neoplastic progression (7, 8, 34). Radiation exposure alters the expression of many genes involved in tissue processes such as proteases, growth factors, cytokines, and adhesion proteins, which supports the view that carcinogenesis could compromise tissue integrity by altering the flow of information among cells (35, 36). Indeed, our recent experimental studies demonstrate that multicellular architecture can be dominant over genomic change in terms of malignant cellular behavior (18, 37, 38). In these studies, breast cancer cells treated with β1 integrin function-blocking antibodies revert from disorganized colonies to organized acinar-like colonies that are characterized by restoration of cytoskeletal organization, cell–cell and cell–ECM interactions, and reduced tumorigenecity (18). Small molecule inhibitors can also be used to cooperatively block aberrant signaling and revert tumorigenic behavior (37, 38). These data, and others in hematopoetic cancers (39), suggest that cancer can be controlled by reestablishing appropriate contacts from the ECM and stroma via outside-in signaling.

Although radiation can acutely regulate E-cadherin and α-catenin levels (40), as well as integrin expression (41), in our studies the phenotype is exhibited by the daughters of irradiated cells several generations after radiation exposure. The redistribution of β1 integrin (Fig. 1) in daughters of irradiated cells was accompanied by increased protein determined by immunoblotting (Fig. 4). In contrast, even though TGF-β treatment decreased E-cadherin and β-catenin protein levels (Fig. 4), localization of E-cadherin and β-catenin immunoreactivity was only affected in double-treated 3D rBM colonies (Fig. 2). Immunostaining can reveal protein access or conformation as well as protein abundance. Preliminary studies suggest that the cell-adhesion proteins of irradiated cells have altered cytoskeletal associations (A.C.E. and M.H.B.-H., unpublished data).

Based on studies in mouse mammary gland, we have proposed that the action of radiation as a carcinogen is augmented by its ability to compromise signaling from the stromal microenvironment (42). A functional test of this concept is provided by our experiments showing that tumorigenesis is increased 4-fold when unirradiated preneoplastic mammary epithelial cells are transplanted to an irradiated mammary stroma (9). One of the most rapid and sensitive responses in the irradiated tissue is the activation of TGF-β (43). TGF-β has a paradoxical effect during carcinogenesis in that it suppresses tumorigenesis but promotes neoplastic progression (44–46). Overexpression of active TGF-β can also induce an epithelial-mesenchymal phenotypic transition during progression *in vivo* (47). In culture, this phenotype is characterized by loss of E-cadherin, acquisition of mesenchymal cytoskeletal features, and increased cell motility and invasion (48). In our experiments, this effect of TGF-β appears to be augmented by preirradiation of the cells. Similarly, the loss of E-cadherin after very low IR doses may further compromise this essential mediator of cell–cell adhesion in preneoplastic breast cells that already have less E-cadherin (49, 50), and could promote progression.

The loss of cell polarity and multicellular organization exhibited by the progeny of irradiated cells suggest that radiation exposure could promote malignant progression by pathways initially independent of mutational mechanisms. Consistent with this postulate is the observation that colonies from irradiated HMEC contain more cells, indicating that decreased cell–cell communication resulted in loss of contact inhibition and greater proliferation. The events leading to disrupted multicellular organization in the progeny of irradiated HMEC could also contribute to genomic instability. Radiation-induced genomic instability evidenced by increased frequency of mutation and cell death occurs in the progeny of irradiated bone marrow (51, 52) and epithelial cell culture (53). The disruption of cell contacts could permit abnormal cells to persist (54) or dysregulate genome stability functions. Inappropriate mammary expression of an activated metalloprotease in transgenic mice that disrupts cell–ECM interactions and cleaves E-cadherin leads to genomic instability (D. Radisky and M.J.B., unpublished data) and mammary tumors (55, 56).

Here we show that IR can promote phenotypic progression by affecting pathways that inhibit the ability of daughter cells to interact with each other and the microenvironment. Agents designed to protect irradiated tissue from disruption of cell–cell communication (57), or those that can reverse the irradiated phenotype, could provide a means of impeding its downstream carcinogenic potential.

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Research article

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Id-1 is not expressed in the luminal epithelial cells of mammary glands

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Abstract

Background: The family of inhibitor of differentiation/DNA binding (Id) proteins is known to regulate development in several tissues. One member of this gene family, Id-1, has been implicated in mammary development and carcinogenesis. Mammary glands contain various cell types, among which the luminal epithelial cells are primarily targeted for proliferation, differentiation and carcinogenesis. Therefore, to assess the precise significance of Id-1 in mammary biology and carcinogenesis, we examined its cellular localization *in vivo* using immunohistochemistry.

Methods: Extracts of whole mammary glands from wild type and Id-1 null mutant mice, and tissue sections from paraffin-embedded mouse mammary glands from various developmental stages and normal human breast were subjected to immunoblot and immunohistochemical analyses, respectively. In both these procedures, an anti-Id-1 rabbit polyclonal antibody was used for detection of Id-1.

Results: In immunoblot analyses, using whole mammary gland extracts, Id-1 was detected. In immunohistochemical analyses, however, Id-1 was not detected in the luminal epithelial cells of mammary glands during any stage of development, but it was detected in vascular endothelial cells.

Conclusion: Id-1 is not expressed in the luminal epithelial cells of mammary glands.

Keywords: human, Id-1, immunohistochemistry, mammary glands, mouse

Introduction

Inhibitor of differentiation/DNA binding (Id) proteins belong to a subfamily of helix–loop–helix (HLH) proteins. Four mammalian members of this family (Id1–Id4) have been identified. The distinguishing characteristic of Id proteins is that, unlike the basic HLH proteins, they do not contain a basic DNA binding domain. Nevertheless, they can regulate cell functions primarily by dimerization with other transcriptional regulators, principally basic HLH proteins.

There is extensive documentation that Id proteins promote cell proliferation and negatively regulate differentiation. High levels of Id gene expression have also been observed in tumor cell lines derived from different tissues [1,2]. In accordance with this, one of the members of this gene family (Id-1) has been shown to promote proliferation and to inhibit functional differentiation of mouse mammary epithelial cells (SCp2 cells), maintained in cell culture [3].

The normal mammary gland is composed of several cell types, but it is the luminal epithelial cells lining the inside of the ducts and the lobules that are primarily targeted for proliferation, differentiation and carcinogenesis. Therefore, to assess the precise significance of any regulatory factor in mammary biology and its significance to carcinogenesis, it is essential to examine its cellular localization *in vivo*. This is particularly important in the case of ubiquitously expressed proteins, such as Ids. Accordingly, in the present study we examined the *in situ* localization of Id-1

HLH = helix–loop–helix; Id = inhibitor of differentiation/DNA binding.

in normal mammary glands, and we report that Id-1 is not expressed in the luminal epithelial cells.

Materials and methods

The source of mammary tissues, FVB and BALB/c strains of mice, used for developmental studies were as follows: pubertal (6 weeks old), adult nulliparous (12 weeks old), early pregnant (6 days gestation), lactating (day 7, postpartum), and postlactational involution (3 days after pup removal). Id-1 null mutant mice (129Sv/C57BL) have been described previously [4]. For these null mutant mice, the corresponding strain of wild type mice was used as a control. The mice were housed and cared for in accordance with the National Institutes of Health guide to humane use of animals in research.

For immunoblot analyses, tissues were frozen in liquid nitrogen and stored at −70°C until use. For immunohistochemical analyses, mammary glands were fixed in 4.7% buffered formalin, dehydrated, embedded in paraffin and cut into 5 µm thick sections. Tissue sections from paraffin-embedded normal human breast were kindly provided by Dr Paul Yaswen.

Source of anti-Id-1 antibody

An anti-Id-1 rabbit polyclonal antibody (C-20) and the peptide used for the generation of the antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunoblot analyses

Protein extracts were prepared from mammary tissues by homogenization in lysis buffer (50 mM Tris–HCl [pH 8.0], 125 mM NaCl, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 1 mM phenylmethylsulfonyl fluoride) containing the protease inhibitors leupeptin, pepstatin and aprotinin, each at a final concentration of 1 µg/ml. The homogenates were sonicated, centrifuged at 110 *g*, and the pellets were discarded. Protein concentrations in the supernatants (lysates) were determined by DC protein assay (BioRad, Hercules, CA, USA). Aliquots of mammary gland lysates equivalent to 40 µg protein were subjected to electrophoresis through 10–20% gradient gels and transferred to nitrocellulose membranes. The membranes were blocked with 10% nonfat powdered milk prior to treatment with the primary antibody. The blots were subsequently washed and treated with appropriate secondary antibodies. The resulting antigen–antibody complexes were detected by the ECL system (Amersham Pharmacia biotech, Chalfont, UK).

Analysis for *in situ* localization of Id-1

For immunohistochemistry, tissue sections were deparaffinized, rehydrated, and soaked in antigen unmasking solution (Vector, Burlingame, CA, USA), The sections were then heated in the microwave oven for 21 min to reveal antigens. The sections were incubated with Immuno Pure Peroxidase Suppressor (Pierce, Rockford, IL, USA) to quench the endogenous peroxidase for 1 hour. The Biotin/Avidin blocking kit (Vector) was then used to block the nonspecific background. The antigen–antibody complexes were identified using the Universal DAKO LSAB2-labeled streptavidin–biotin peroxidase kit (DAKO, Carpinteria, CA, USA). The sections were counterstained with Mayer's hematoxylin solution (DAKO).

Results

Validation of the antibody

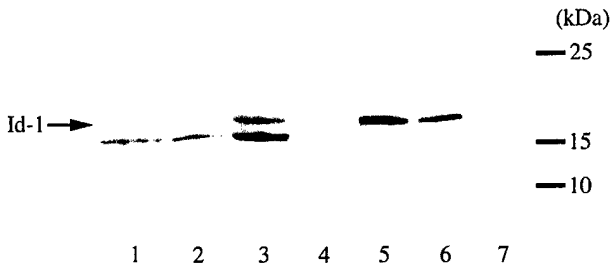
The Id-1 antibody used in the present studies (C-20) was the same as that used previously in immunoblot analyses for demonstrating Id-1 expression in various tissue/cell extracts, including mammary cells [3,5–8]. As shown in Figure 1, in immunoblot analyses of whole mammary gland extracts of wild type mice (lanes 1 and 3 corresponding to 129Sv/C57BL and FVB, respectively), two immunoreactive bands were detected in the region of 15–20 kDa. Among these, the top band was absent in extracts prepared from mammary glands of Id-1 null mutant mice (Fig. 1, lane 2), indicating that it corresponded to Id-1. In contrast, in extracts of wild type mouse testis, as reported previously [5], a single immunoreactive band was detected (Fig. 1, lane 4) and this corresponded to the top band in mammary extracts. Similarly, only the top band was detected in extracts of wild type mouse uterus and intestine (Fig. 1, lanes 5 and 6, respectively). Both the top and the bottom bands, detected in mammary extracts of wild type mice, were absent when blots were incubated with Id-1 antibody in the presence of the peptide used for generation of the antibody (Fig. 1, lane 7). These bands were also absent with the deletion of the primary antibody (data not shown). These observations thus confirmed that the C-20 antibody was capable of detecting Id-1, and also demonstrated its presence in whole mammary gland extracts.

The C-20 antibody is known to detect Id-1 in paraffin-embedded tissue sections and has been used successfully for the analyses of Id-1 in sertoli cells of testis by immunohistochemistry [5]. Nevertheless, we verified the ability of the C-20 antibody to detect Id-1, with fidelity, in immunohistochemical analyses. To achieve this we analyzed the vasculature of the developing brain, since Id-1 gene expression has been demonstrated previously in this tissue, by *in situ* hybridization [9]. Immunoreactivity was detected in the nuclei of the vasculature of the developing brain, as shown in Figure 2A,B, and the pattern of immunostaining was similar to that observed for Id-1 gene expression.

Id-1 is not detectable in luminal epithelial cells of mammary glands

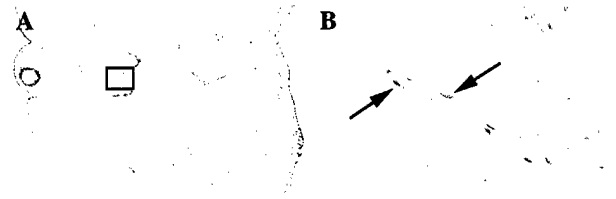
We next examined the *in situ* localization of Id-1 in mammary glands. As shown in Figure 3A, immunoreactiv-

Figure 1



Immunoblot analyses for Id-1 in various mouse tissues. Lysates were prepared from various mouse tissues and analyzed for Id-1, using C-20 antibody, as described in the text. The positions of the molecular weight standards (kDa) are shown on the right. Lanes 1 and 3, wild type mammary glands (129Sv/C57BL and FVB, respectively); lane 2, mammary glands from Id-1 null mutant mouse; lanes 4–6, testis, uterus and intestine from wild type mice, respectively; lane 7, mammary gland extract of wild type mice in which treatment with the primary antibody was performed in the presence of the blocking peptide.

Figure 2



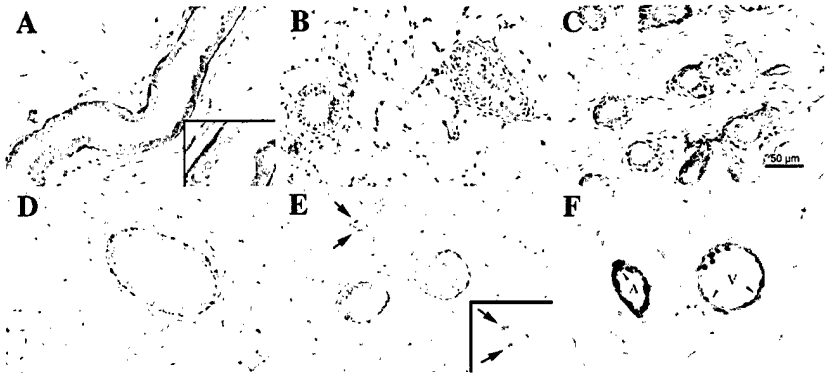
Immunohistochemical analyses for Id-1. **(A), (B)** The immunoreactivity in the vasculature (arrows) of the developing brain (inset, B) at day 12.5 of gestation. Original magnification, (A) 50 ×, (B) 400 ×.

ity was not detected either in the luminal epithelial cells or in the stroma of mammary glands of adult nulliparous females. Immunoreactivity was detected, however, in the cytoplasm of myoepithelial cells, and this was completely abolished in the presence of the blocking peptide used for the generation of Id-1 (C-20) antibody (Fig. 3B). Nevertheless, the staining observed in the myoepithelial cells did not appear to be specific to Id-1 since it was also present in mammary myoepithelial cells of Id-1 null mutant mice (compare Fig. 3D and 3E). Similar to mouse mammary glands, Id-1 immunoreactivity was also not observed in luminal epithelial cells of normal human mammary glands, but was detected in the myoepithelial cells (Fig. 3C).

The fact that Id-1 could be detected in mouse mammary tissue extracts by immunoblot analyses but was undetectable in mammary cells by immunohistochemical analyses led us to consider that the Id-1 detected in tissue extracts might have been derived from nonmammary cells. A potential source was vascular endothelial cells since Id-1 is also expressed in the blood vessels outside the central nervous system [9]. Immunoreactivity was detected in the vascular endothelial cells in mammary glands of wild type adult mice (Fig. 3F), but not in the cells of Id-1 null mutant mice (Fig. 3E, inset).

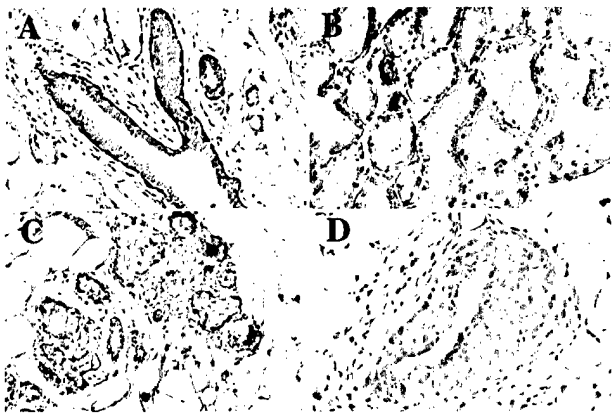
It is well known that mammary epithelial cells of adult nonpregnant females, for the most part, are mitotically quiescent and proliferate extensively only with the onset of pregnancy [10]. Therefore, to examine whether the lack of expression of Id-1 in the luminal epithelial cells was related to its quiescent state, we also analyzed the tissues from early pregnant females. The patterns of immunostaining in mammary glands did not change either during pregnancy

Figure 3



Immunohistochemical analyses for Id-1 in mammary glands. All tissue sections were treated with the primary antibody (except (B), which was incubated with both the primary antibody and the blocking peptide) and processed as described in the text. **(A), (B)** Adult nulliparous wild type mice (FVB), **(C)** normal human mammary gland, **(D)** adult nulliparous wild type mice (129Sv, C57BL/6) corresponding to the strain of Id-1 null mutant mice, **(E)** arrows showing the vascular endothelial cells, and **(F)** vascular endothelial cells (arrows) in an artery (A) and a vein (V) in mammary glands of wild type mice. Original magnification: (A)–(E) 400 ×. Insets of (A) and (E), higher magnification (630 ×).

Figure 4



Immunohistochemical analyses for Id-1 in mouse mammary glands during various phases of development. Mammary glands from **(A)** early pregnant, **(B)** lactating, **(C)** lactational involuting and **(D)** pubertal mice were analyzed for the presence of Id-1 as described in the text. Note that, in all the mammary ducts, immunoreactivity is present only in myoepithelial cells and not in the luminal cells. Immunoreactivity is also not detected in the terminal end bud of the pubertal mouse. Original magnification, 400 ×.

(Fig. 4A), during lactation (Fig. 4B) or during postlactational involution (Fig. 4C). As such, immunoreactivity was still confined to the cytoplasm of the myoepithelial cells and was not detected in luminal epithelial cells. Similar results were also obtained with tissues isolated from the Balb/c strain of mice (data not shown), the strain from which SCp2 cells had been derived [11]. In addition to pregnancy, mammary glands also proliferate extensively at the onset of puberty and, in these glands, the site of intense mitotic activity resides in specialized structures called the terminal end buds [12]. In terminal end buds of pubertal females, immunoreactivity was not detected either in the body cells or the cap cells (Fig. 4D) present at the tips.

Discussion

In the present report, we have demonstrated that Id-1 is not detectable in the luminal epithelial cells of both mouse and human mammary glands. Our inability to detect Id-1 in these cells is not related to the source of the antibody or techniques since it was possible to detect Id-1 in vascular endothelial cells by immunohistochemistry and via immunoblot analyses of several mouse tissue extracts, including mammary glands. The fact that Id-1 is detected in mammary tissue extracts by immunoblot analyses but not by immunohistochemical analyses therefore indicates that Id-1 detected in whole tissue extracts is derived from non-mammary cells. This argument is supported by our demonstration that Id-1 is expressed in vascular endothelial cells of mammary glands. Furthermore, in contrast to Id-1, we can detect Id-2 in the luminal epithelial cells of mouse mammary glands (N Uehara, Y-C Chou and G Shyamala, unpublished observation) in which Id-2 gene expression has been demonstrated by *in situ* hybridization [13]. Based on all these observations, we conclude that Id-1 is not expressed in the luminal epithelial cells of mammary glands.

Our studies also demonstrate clearly that in mice, regardless of the strain or the developmental stage, Id-1 is not detectable in the luminal epithelial cells; this included both puberty and early pregnancy, when mammary glands undergo extensive proliferation. Accordingly, our present observations do not support the previous suggestion that, in mammary epithelial cells, Id-1 is a positive and a negative regulator of proliferation and of differentiation, respectively [3]. In turn, the observations also emphasize that the postulated roles for Id-1, using various cell culture models, may not be applicable to all cell types, particularly *in vivo* [2]. This is exemplified by the fact that much of the information demonstrating various regulatory roles for Id proteins have used fibroblasts and, as shown here, mammary fibroblasts do not express Id-1.

Finally, the detection of 'non-Id-1' immunoreactivity in immunoblot analyses and in myoepithelial cells, with immunohistochemistry, requires comment. It is clearly not due to an abundant nonspecific protein since the immunoreactivity associated with the myoepithelial cells is quite discreet. Indeed, the immunoreactivity associated with the myoepithelial cells is completely abolished by the blocking peptide (Fig. 2). It is also not due to Id-2 or Id-3, since this antibody is specific to an epitope in the carboxy terminus of Id-1 and has no crossreactivity with these proteins [6,8]. Furthermore, the nonspecific band detected in immunoblots appears to have some specificity for mammary glands since, to date, we have not detected this band in other mouse tissue extracts. Also, the nonspecific band is eliminated upon exposure to the blocking peptide.

Myoepithelial cells express a number of proteins but several of these are also expressed by luminal epithelial cells [14]. As such, very few proteins are expressed exclusively in the myoepithelial cells, the most prominent one being alpha smooth muscle actin [15]. It is, however, unlikely, that the 'non-Id-1' immunoreactivity associated with myoepithelial cells is alpha smooth muscle actin since it has minimal homology to Id-1 and, in particular, to the peptide used for the generation of the antibody. It is therefore most probably due to some other smooth muscle cell-specific protein capable of recognizing the epitope on the Id-1 (C-20) antibody. Identification of this protein can thus lead to establishing another myoepithelial cell-specific marker, which in turn can contribute to our current understanding of the biology of mammary myoepithelial cells.

Conclusion

Id-1 detected in whole mammary gland extracts is not derived from luminal epithelial cells.

Competing interests

None declared.

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